Bohn, Brent 1031

From: Powers, Christina
Sent: Monday, July 07, 2014 12:45 PM
To: Gift, Jeff
Cc: Lee, Janice; Cowden, John; Powers, Christina
Subject: iAs Dose Response and MOA
Attachments: 2014 Budinsky MOA DR Framework AHR Crit Rev Tox.pdf

Hi Jeff,

The attached article might be of interest in relation to considering MOA in dose-response analyses for arsenic. The article is not specific to arsenic (TCDD as an AHR ligand is the example), but the authors present an argument/ approach for using early key events in a MOA in developing a POD. The article is a bit long, but I'm passing it along in case you're interested in taking a look at the "Dose-Response" section (p. 104-106).

As always, I'm happy to discuss further if it's helpful. Hope you had a great holiday weekend and the week is going well!

Best,
Christy

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REVIEW ARTICLE

Mode of action and dose–response framework analysis for receptor-mediated toxicity: The aryl hydrocarbon receptor as a case study

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Abstract

Dioxins and dioxin-like compounds are tumor promoters that cause liver cancer in rats and mice. The aryl hydrocarbon receptor (AHR) has been implicated as a key component in this tumor promotion response. Despite extensive knowledge of the toxicology of dioxins, no mode of action (MOA) hypothesis for their tumorigenicity has been formally documented using the Human Relevance MOA framework developed by the International Programme on Chemical Safety (IPCS). To address this information gap, an expert panel was convened as part of a workshop on receptor-mediated liver tumorigenicity. Liver tumors induced by ligands of the AHR were assessed using data for dioxins and related chemicals as a case study. The panel proposed a MOA beginning with sustained AHR activation, eventually leading to liver tumors via a number of other processes, including increased cell proliferation of previously initiated altered hepatic foci, inhibition of intrafocal apoptosis and proliferation of oval cells. These processes have been identified and grouped as three key events within the hepatocarcinogenic MOA: (1) sustained AHR activation, (2) alterations in cellular growth and homeostasis and (3) pre-neoplastic tissue changes. These key events were identified through application of the Bradford-Hill considerations in terms of both their necessity for the apical event/adverse outcome and their human relevance. The panel identified data supporting the identification and dose–response behavior of key events, alteration of the dose–response by numerous modulating factors and data gaps that potentially impact the MOA. The current effort of applying the systematic frameworks for identifying key events and assessing human relevance to the AHR activation in the tumorigenicity of dioxins and related chemicals is novel at this time. The results should help direct future regulatory efforts and research activities aimed at better understanding the potential human cancer risks associated with dioxin exposure.

Keywords

Dioxin, dose–response assessment, human relevance framework, key events, liver cancer, mode of action, modulating factors, TCDD

History

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Introduction

Background and rationale for evaluation

The aryl hydrocarbon receptor (AHR) pathway is one of the most studied in toxicology. It mediates the biological activity of dioxin-like chemicals that include 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent AHR agonist, as well as several less potent dioxin-like chemicals, including 6 other polychlorinated dibenzo-*p*-dioxins (PCDDs), 10 polychlorinated dibenzofurans (PCDFs) and 12 coplanar polychlorinated biphenyl (PCBs) congeners. Controversy has swirled around the carcinogenicity of dioxin-like chemicals in rodents regarding whether the dose–response is best understood as a linear non-threshold or a non-linear threshold[1] phenomenon, and, not least, the human relevance of this rodent response (JEFCA, 2003; USEPA, 2010).

Interest in the mechanistic basis for AHR action arises from the demonstrated relationship between activation of this receptor, carcinogenicity and concerns over occupational and environmental exposures to TCDD and other dioxin-like chemicals. TCDD produces hepatocellular adenomas and carcinomas, cholangioma and cholangiocarcinoma, predominantly in female rats (Table 1) (Hailey et al., 2005; Kociba et al., 1978; NTP, 2006a; Walker et al., 2006). Tumor incidence in male rats is considerably less for both tumor types. Supplemental figure shows a comparison of adenomas and carcinomas in the livers of male and female rats (Kociba et al., 1978). This gender difference suggests a relationship between the AHR and the estrogen receptor that has subsequently been explored (Ahmed et al., 2009; Matthews & Gustafsson, 2006; Wihlen et al., 2009). In mice, both males and females develop liver tumors following TCDD treatment; however, in contrast to rats, male mice display higher rates of both adenomas and carcinomas than do females (Supplemental Figure). Lifetime cancer bioassays in rodents have been completed for other dioxins, furans and dioxin-like PCBs including 2,3,4,7,8-pentachlorodibenzofuran, a mixture of hexachlorodibenzo-*p*-dioxins, and PCB 126 and 118 (NTP, 1980, 1982, 2006a,b,c, 2010). The studies are notable for a close linkage between histopathology and tumor development and the unusual extent and presentation of hepatic lesions (Goodman & Sauer, 1992; Hailey et al., 2005). Dioxins also cause lung tumors and tumors of the oral mucosa in rodents (NTP, 2006a) as well as facial skin squamous cell carcinomas in hamsters (Rao et al., 1988). In recent years, regulatory attention and risk assessment evaluations have been applied to characterize the carcinogenicity of TCDD. As a result of this scrutiny and research focus, TCDD serves as the prototypical ligand for the AHR, and provides important lessons as a non-mutagenic case study for a tumorigenic mode of action (MOA) evaluation.

Pitot et al. (1987) suggested that TCDD acts as a tumor promoter because it lacked covalent interaction with DNA and failed to exhibit mutagenic activity (Poland & Glover, 1979; Wassom et al., 1977). This suggestion led to numerous tumor promotion studies with dioxins using various initiation and/or partial hepatectomy protocols[2] (Buchmann et al., 1994; Dragan et al., 1992; Luebeck et al., 2000; Maronpot et al., 1993; Pitot et al., 1987; Schrenk et al., 1994; Stinchcombe et al., 1995; Teeguarden et al., 1999; Tritscher et al., 1992, 1995; Walker et al., 1998; Wyde et al., 2001a) (see Supplemental information for a summary table of the published dioxin initiation–promotion studies). Increases in the number and volume of phenotypically altered (e.g. GSTP-positive) foci derived from these results have been quantitatively modeled (Andersen & Conolly, 1998; Andersen et al., 1993, 1995; Bock & Kohle, 2005; Conolly & Andersen, 1997; Kim et al., 2003; Mills & Andersen, 1993; Moolgavkar et al., 1996; Portier et al., 1993, 1996). Several studies have produced data that show evidence of non-linearity for increased volume fraction of altered hepatic foci as a measure of tumor promotion (Maronpot et al., 1993; Pitot et al., 1987; Teeguarden et al., 1999; Viluksela et al., 2000). Other stochastic tumor-promotion models have been used to predict low-dose cancer risks at levels well below apparent

[1]A threshold is defined as a non-linear dose–response that is a range of exposures from zero to some finite value with no detectable expression of a toxic effect, and the threshold of toxicity is where the effects (or their precursors) become quantifiable.

[2]It is important to recognize that the nitrosamine initiation and partial hepatectomy design likely create somewhat spurious dose–response information by artificially stimulating the development and clonal expansion of altered hepatic foci before the promotion effect of sustained AHR activation was added. Hence, dose–response estimates derived from these initiation–promotion studies must account for this factor. Altered foci do occur in TCDD-treated non-initiated rats, but at a very low rate (Brix et al., 2005; Maronpot et al., 1993; McMartin et al., 1992; Newsholme & Fish, 1994).

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Table 1. Summary of key chronic tumor bioassays in rodents treated with TCDD.

Study	Dose (ng/kg day) and liver tumors					
Rats						
Kociba et al., 1978; Spartan Sprague Dawley†	**0**	**1**	**10**	**100**		
Female	2/86	1/50	9/50	18/45		
Male	2/85	0/50	0/50	1/50		
NTP, 1982; Osborne Mendel‡	**0**	**1.4**	**7.1**	**71**		
Female	5/75	1/49	3/50	14/49		
Male	0/74	0/50	0/50	3/50		
NTP, 2006a; Harlan Sprague Dawley	**0**	**2.1**	**7.1**	**16**	**33**	**71**
Female Only						
Cholangiolar	0/53	0/54	0/53	1/53	4/53	25/53
Adenoma	0/53	0/54	0/53	0/53	1/53	13/53
Hepatocholangioma	0/53	0/54	0/53	0/53	0/53	2/53
Mice						
NTP, 1982; B6C3F1‡	**0**	**6.7**	**67**	**330**		
Female	3/73	6/50	6/48	11/47		
	0	**1.7**	**8.3**	**83**		
Male	15/73	12/49	13/49	27/50		
Della Porta et al., 1987; B6C3	**0**	**180**	**360**			
Female§	3/49	16/42	20/48			
	0	**180**	**360**			
Male§	15/43	26/51	43/50			

†Number of animals with tumors/total number of animals in the study.
‡Number of tumor-bearing animals/number of animals examined at site; neoplastic nodule or hepatocellular carcinoma.
§Combined liver carcinoma and adenoma.

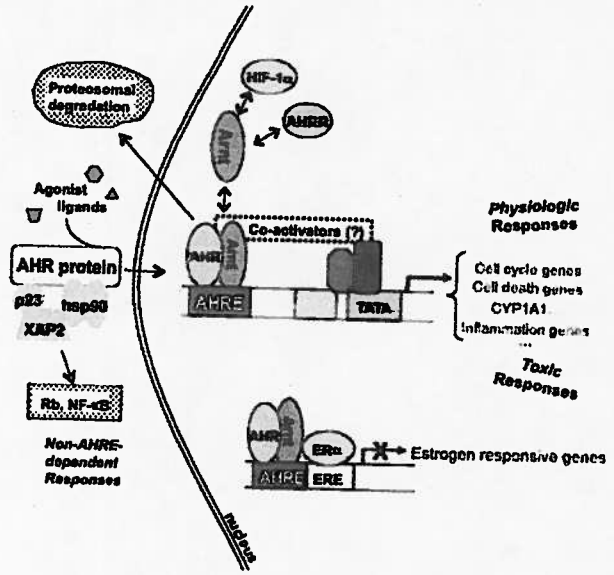

Figure 1. AHR Binding of the AHR-ARNT-ligand complex to the AHRE initiates recruitment of transcription factors and other co-regulatory proteins leading ultimately to modulation of gene expression (Modified and reprinted from Critical Reviews in Eukaryotic Gene Expression, Volume 18, Gasiewicz TA, Henry EC, Collins LL, Expression and activity of aryl hydrocarbon receptors in development of cancer, Pages 279–321, Copyright (2008), with permission from Begell House, Inc.).

effect thresholds (Moolgavkar et al., 1996; Portier et al., 1996). These models found increases in the number of initiated foci following TCDD administration; however, increased foci number is generally an effect of the initiator, whereas increases foci volume or cell number is an effect of TCDD as a promoter of cell division and apoptosis. In the examination of the MOA, sustained AHR activation acts on both normal liver cells and focal tissue to bring about clonal expansion and conversion of altered hepatic foci into liver tumors.

The potential role of persistent AHR activation as a MOA for carcinogenesis has been the focus of a great deal of research (Bock & Kohle, 2006; Cole et al., 2003; Gasiewicz et al., 2008; Knerr & Schrenk, 2006). Many published *in vivo* and *in vitro* studies support the current understanding of how dioxins act as tumor promoters, i.e. through sustained activation of the AHR that, in part, promotes cell division and prevents initiated cells from undergoing apoptosis (see further discussion of this topic in the "Results" section below) (Chopra & Schrenk, 2011; Gasiewicz et al., 2008; Mitchell & Elferink, 2009). The biological responses to AHR ligands are complex, and the role of this receptor in normal biology is only partly understood (Gasiewicz et al., 2008). However, this understanding is growing and the role of the AHR in inflammatory and allergic diseases, diabetes and human cancer is becoming apparent (Keates et al., 2000; Kerkvliet et al., 2009; Schulz et al., 2011).

Molecular biology of the AHR: regulatory role and species differences

Aryl hydrocarbon receptor-mediated transcription begins with the ligand binding to a cytosolic AHR complex that includes the AHR, heat shock protein 90 dimer (Hsp90), p23 and X-associated protein 2 (XAP2) (Figure 1). Ligand binding elicits a conformational change in the AHR that results in cytoplasmic-to-nuclear translocation, shedding of bound proteins and interaction with the aryl hydrocarbon nuclear translocator protein (ARNT). The AHR-ARNT-ligand complex recognizes specific DNA sequences (i.e. AHR-responsive elements or AHREs) in the regulatory regions of genes modulated by the AHR. Binding of the AHR-ARNT-ligand complex to the AHRE initiates recruitment of other transcription factors and co-regulatory proteins and modulation of gene expression (Abel & Haarmann-Stemmann, 2010; Dietrich & Kaina, 2010; Furness & Whelan, 2009; Gasiewicz et al., 2008) (Figure 1). The most extensively characterized genes regulated by the AHR are xenobiotic metabolizing enzymes but it is important to emphasize that the AHR functions as a transcription factor with impact on important cellular responses beyond enzyme induction. It is important to note that the induction of xenobiotic metabolizing enzymes are not directly associated with key events; rather, they serve effectively as biomarkers of exposure.

Current descriptions of AHR-mediated transcriptional regulation are simplifications; not only do differences in ligand potency affect the transcriptional response, but also nuances in biological context alter the effects of various ligands. For example, agonist or antagonist responses to different ligands are clearly dependent on differences in ligand binding affinity, efficacy, conformation of the ligand–

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receptor complex, and the relative ability of the ligands to be metabolized. Differences in types of AHREs, flanking nucleotide sequences and positioning relative to other transcription factor binding sites all influence the efficacy and sensitivity of AHR-mediated transcription. The promiscuous role of transcription factors, co-regulators and small RNAs in control of transcriptional activity adds even more complexity, suggesting control of gene expression may be exquisitely sensitive to biological context as well as the nature of the ligand (Abdelrahim et al., 2003; Chute et al., 2010; Hankinson, 2005; Kim & Stallcup, 2004; Kollara & Brown, 2006; Mukai et al., 2010; Nakata et al., 2009; Nishiumi et al., 2007; Partch et al., 2009; Taylor et al., 2009; Turgeon et al., 2004; Wang et al., 2004; Watabe et al., 2010; Zhang et al., 2003).

Target genes regulated by the AHR may not be well conserved across strains and species (Boutros et al., 2011; Boverhof et al., 2006; Carlson et al., 2009; Silkworth et al., 2008; Wu et al., 2008). For example, although the overall domain structure of the AHR is conserved across species in general (Hahn et al., 2006), differences in sequence at the ligand binding and C-terminal transactivation domains appear to play a distinct role in determining species differences in both the nature of the response and the dose-dependency of the response (Connor & Aylward, 2006; Ema et al., 1994; Franc et al., 2008; Gasiewicz et al., 2008; Kumar et al., 2001; Moriguchi et al., 2003; Pohjanvirta et al., 1999; Suzuki & Nohara, 2007). Since the discovery of the AHR, many publications have documented species- and tissue-specific differences in its expression, as well as differing responses to a variety of ligands and conditions (Hankinson, 2005). Factors mediating these differences include: (1) AHR degradation by ubiquitin/proteasome-mediated processes, (2) relative expression of, and interaction with, the AHR-repressor (AHRR) protein, (3) regulation of AHR expression by developmental (epigenetic) and tissue-specific factors and (4) regulation of activity by the cell cycle or differentiation state (Abel & Haarmann-Stemmann, 2010; Davarinos & Pollenz, 1999; Franc et al., 2001; Furness & Whelan, 2009; Hahn et al., 2009; Nakata et al., 2009; Pollenz, 2002; Pollenz & Barbour, 2000; Roberts & Whitelaw, 1999).

The AHR may elicit responses through mechanisms that are not AHRE-dependent, but dependent on the direct interaction with other proteins such as NF-κB subunits, estrogen receptors and retinoblastoma protein (Elferink et al., 2001; Ge & Elferink, 1998; Hayes et al., 2009; Kim et al., 2000; Matthews et al., 2005; Puga et al., 2009; Tian, 2009; Tian et al., 1999). For example, in a number of non-liver and liver cell lines, non-genomic responses to TCDD-induced AHR activation have been reported, such as inflammation via cytosolic phospholipase A2 and cyclooxygenase-2 activation (Matsumura, 2009). However, the use of mouse models that have mutations in the AHR nuclear localization sequence and/or the DNA-binding domain suggest that many toxic responses depend on the ability of the AHR to translocate to the nucleus and bind AHREs (Bock & Kohle, 2006; Bunger et al., 2003; Flaveny et al., 2010; Murray et al., 2005, 2010).

The role of the AHR as a mediator of adverse effects needs to be considered within the context of its normal physiological functions (Abel & Haarmann-Stemmann, 2010; Congiu et al., 2009; Denison & Nagy, 2003; Furness & Whelan, 2009; Gasiewicz et al., 2008; Le Vee et al., 2010). Endogenous ligand candidates (e.g. kynurenine, kynurenic acid and indoles) and characteristics of AHR null-allele mice demonstrate a number of cell and tissue processes in which the AHR is required for various normal physiological functions, including T-cell development, inflammatory processes, growth and regulation of reproductive tissues, angiogenesis, neurological development and function, and bone marrow development and maintenance (Akahoshi et al., 2009; Connor et al., 2008; Denison & Nagy, 2003; Gasiewicz et al., 2010; Gomez-Duran et al., 2009; Head & Lawrence, 2009; Hernandez-Ochoa et al., 2009; Ishimura et al., 2009; Kerkvliet, 2009; Marshall & Kerkvliet, 2010; Nguyen et al., 2010; Opitz et al., 2011; Schroeder et al., 2010; Simones & Shepherd, 2011; Singh et al., 2009). Perturbations in several of these processes have been identified as "hallmarks of cancer" (Hanahan & Weinberg, 2011).

Despite substantial risk assessment attention being devoted to TCDD and related chemicals, published reviews have not rigorously applied the human relevance MOA framework in its entirety to the evaluation of the underlying MOA (Bock & Kohle, 2005; Kohle et al., 2008). Here, we present the summary findings of an expert panel convened to evaluate the basis for the liver tumorigenicity MOA mediated through AHR activation using TCDD and other dioxin-like chemicals as model ligands (TERA, 2010). This effort was initiated as an approach to further develop and present updated thinking on the MOA for AHR-mediated liver tumor formation and as a tool for integrating the vast literature on the AHR. Workshop panel members were aware of the risk assessment and policy implications of this work; hence, there is necessarily some discussion of risk assessment policy in this article – most specifically pertaining to the use of this MOA to determine whether linear or non-linear models should be used for low-dose extrapolation (e.g. Simon et al., 2009). As noted in EPA's *Guidelines for Carcinogen Risk Assessment* (USEPA, 2005), consideration of MOA is the centerpiece of any carcinogenic risk assessment. Moreover, in the context of the goals of the workshop, the AHR case study described in this manuscript was one of three receptors studied to evaluate the applications of, and potential refinements to, current iterations of MOA, human relevance and dose–response assessment frameworks when applied to chemicals for which much is known about the underlying molecular toxicology.

Methods

Literature identification and selection process

The extant literature on the tumorigenic MOA and mechanism of TCDD and AHR activation is immense. To provide a manageable approach for the data evaluation at a 2-day expert workshop, literature identification and selection procedures were used to ensure a focus on the most relevant data sources. Key elements of the literature management approach included the following:

- Prior to the workshop, data viewed as likely to have the greatest impact for the MOA evaluation were identified

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through the numerous recent reviews and regulatory assessments. The initial set of references was supplemented by individual expert panel members having expertise in various aspects of AHR biology and TCDD toxicology. A bibliography of key references was developed and copies of individual studies were made available to all panel members via a shared website.

- The scope of the literature review was managed by ensuring that the identified references were relevant to the overall charge of the workshop and that recommended references were sufficiently relevant to the intended risk assessment applications of the analyses. This culling process is embedded in current weight of evidence concepts, e.g. application of "Klimisch" principles (Klimisch et al., 1997). Thus, key papers generally focused on human effects studies, rodent (rat or mouse) *in vivo* studies or *in vitro* studies using relevant cell types (primary liver cells preferred). Moreover, studies using dose-ranges at or below those that cause tumors in rodents were given greater weight. The data on pathological and tumor responses were limited to those impacting the liver. Data on mechanistic or early molecular responses were examined as being reflective of potential key events in the MOA and were identified by individual expert panel members prior to the workshop.
- Discussions held at the workshop yielded suggestions for additional areas for follow-up evaluation. To reflect these suggestions, a supplemental literature search was conducted for the preparation of this manuscript using online databases (PubMed and TOXLINE) managed by the National Library of Medicine. Additional screening reduced the number of new papers to less than 100. These new papers were then culled for relevance to the MOA for dioxin-induced or AHR-mediated liver tumors. Those studies that remained after the culling process were selected for review and possible inclusion in this manuscript.

Mode of action/human relevance and key events/dose–response frameworks

The evaluation of the salient literature was organized around a series of panel discussion questions (Table 2). The discussion questions were designed to elicit a robust expert panel evaluation of the key aspects of current MOA evaluation framework (Boobis et al., 2006, 2009; Cohen et al., 2003, 2004; Holsapple et al., 2006; Julien et al., 2009; Meek, 2008; Meek et al., 2003; Seed et al., 2005; Sonich-Mullin et al., 2001; USEPA, 2005). In brief, the key aspects addressed:

- Developing and testing alternative hypotheses for the MOA using the Key Events/Dose–Response Frameworks (KEDRF) and the Bradford-Hill considerations for identifying key events (KE), associative events (AE) and modulating factors (ModF) in the various MOAs;
- Testing the human relevance of these MOAs in both a qualitative and quantitative fashion using the MOA/human relevance framework and
- Evaluating the implications of the proposed MOA for understanding the carcinogenic response in rodents.

Prior to the workshop, the Workshop Steering Committee developed unified definitions for KEs, AEs and ModFs and identified the data evaluation framework to be applied for the MOA evaluation. These processes are described in detail in the first manuscript in this series (Andersen et al., in press). In this article, the details of the MOA in rodents will be considered first and then the human relevance of this MOA and the various KEs will be assessed. Because the amount of material reviewed is large, a simple conformance to the formatting of a Human Relevance/MOA framework approach was not possible. However, the intent of this review was to follow the guidelines defined within the framework.

AHR case study panel procedures

In order to apply the framework methodology in a comprehensive fashion to establish a hepatic carcinogenic MOA given the inherent complexity of the cancer process, the workshop panel relied on a multi-disciplinary approach. By bringing together experts in a range of disciplines, most elements supporting the MOA could be discussed, including molecular biology, histopathology, quantitative dose–response modeling and others. AHR panel members were selected to ensure coverage of all key aspects of AHR tumor biology, dioxin toxicology and epidemiology, dose–response assessment and risk assessment (Table 3). The overall panel selection process is described elsewhere (Andersen et al., in press; TERA, 2010). Panel members shared their own scientific opinions and not those of the agencies or organizations with which they were affiliated.

Results

The results of the workshop reflect discussions and analyses of the panel as presented in a formal rapporteur's report on the third day of the overall workshop. The summary statements for each of the discussion questions are presented in Table 2. The data and analyses supporting these conclusions are described in this section.

Mode of action hypothesis

The AHR panel members concluded that TCDD acts via a tumor promotion MOA with sustained near-maximal[3] AHR activation for a significant portion of the life span as the pivotal and initial KE; the apical outcome of this MOA is the late development of female rat liver tumors. At the cell and tissue levels, the other two KEs were: (1) altered focal cell proliferation[4] (i.e. changes in cell division and apoptosis) and

[3]Sustained near-maximal activation is defined for tumor outcomes based on the dose–response relationship between CYP1A induction over time and the occurrence of tumors at several time intervals up to 1 year. CYP1A1 induction between 75% and 90% of the maximal induction corresponded to a 1% tumor response for both hepatic adenoma and cholangiocarcinoma (Simon et al., 2009).

[4]The rate of cell proliferation within any population of cells depends on three parameters: (a) the rate of cell division, (b) the fraction of cells within the population undergoing cell division (growth fraction) and (c) the rate of cell loss from the population due to terminal differentiation or cell death. If the rate of replication outpaces the rate of cell death, then cell proliferation occurs (http://www.ncbi.nlm.nih.gov/books/NBK20860/).

Table 2. Primary charge questions and concluding comments from the AHR case study panel rapporteur report.

Discussion questions (Q) and panel overall conclusions (A) for each
Q1. What is the mode of action (MOA) for AHR-mediated rodent liver tumors for a model AHR activator (e.g. dioxins or related compounds), as evaluated using the IPCS Framework for Human Relevance and the modified Hill Criteria applied to MOA (IPCS and EPA MOA Framework)?
A1. See Figure 1 (MOA).
Q2. What are the *early biological steps* necessary to affect the formation of liver tumors?
A2. The existing molecular biology for gene regulation is sufficiently understood to support *sustained AHR activation* as a key event. The data for this event meets the requirements for the IPCS MOA/HRF. • The human relevance of sustained AHR activation cannot be reasonably excluded (in qualitative or quantitative terms) based on the available data. • Gene expression (based on mRNA or protein levels) and selected enzyme activities in humans and rats support these conclusions.
Q3. Are the existing data sufficient to determine a dose–response relationship for sustained AHR activation?
A3. Existing data for gene expression and enzyme induction (XMEs) are sufficient for quantitative characterization of sustained AHR activation. • The existing concentration and dose–response data for gene expression and enzyme induction are sufficient for dose–response modeling. • *In vivo* and *in vitro* data are available in both humans and rats. • Data are needed on the relationship of induction of specific genes to phenotypic changes.
Q4. Is there an amount of ligand that would be insufficient for sustained activation of the AHR?
A4. Empirical data indicate that some doses/concentrations are too small to produce detectable changes in sustained AHR activation based on sensitive associative events (measured by XME induction). • Apparent NOELs may be identified for CYP1A gene expression and enzyme induction. • The issue of NOEL identification of the early markers of sustained AHR activation can be problematic due to signal-to-noise considerations at low doses. Phenotypic NOAELs may be identifiable.
Q5. Subsequent to sustained AHR activation, what are the fundamental biological changes necessary to cause *downstream non-apical responses*?
A5. See Figure 1 (MOA).
Q6. Are the existing data sufficient for downstream, non-apical key events to determine a dose–response relationship?
A6. The panel concluded that there may be a difference in the relationship of effects seen in hepatocytes and those seen in biliary cells following sustained AHR activation. • There are sufficient data to support DR modeling for hepatocyte effects. • The lack of quantitative dose–response information for biliary cells is a data gap because intermediate events (key, associative, modulating) have not been identified.
Q7. Is there an amount of ligand that would be insufficient for producing detectable induction of these key events or associated biological responses? Has a NOEL been demonstrated for these downstream, non-apical key events?
A7. Dose–response data are sufficient to determine NOELs for non-apical events in hepatic tissues from *in vivo* studies. • Data are not sufficient to determine similar NOELs for adequately demonstrated causal effects in biliary tract cells.
Q8. Does current knowledge of early and downstream, non-apical key events support the choice of appropriate dose–response models for liver tumors induced through sustained AHR activation?
A8. Weight of evidence points to a non-linear model. • At higher doses that induce non-apical phenotypic effects in rats, there is clear evidence of non-linear responses. • At lower doses where only gene or enzyme changes are observed, it is difficult to determine the shape of the low dose–response. • However, the overall WOE for tumor formation via the proposed MOA favors a non-linear response because the underlying MOA is receptor-mediated and based on the shapes of the DR of key events.
Q9. Additional issues that impact the MOA for the AHR: Where alternative MOA hypotheses considered.
A9. Direct DNA reactivity – not observed for the planar AHR ligands. • Reactive oxygen species formation (impact likely only at high doses). • Catechol estrogen formation and indirect genotoxicity unlikely (other roles of estrogen-dependence remain a possibility but mechanisms not demonstrated). • Cytotoxicity and regenerative hyperplasia (impact only at high doses). • Many of these alternatives were considered modulating factors. • The panel explored a variety of possible *mechanisms* of toxicity following from sustained AHR activation and concluded that sustained activation itself was a clear early key event that would capture the constellation of effects induced by multiple mechanisms.
Q10. Additional issues that impact the MOA for the AHR: What are the key data gaps related to support for the proposed MOA?
A10. Stem cells that form both hepatocytes and biliary cells may be targets for TCDD-induced effects. The identity of the initial target cell population not known. • The underlying mechanism for inhibition of apoptosis is not known. • Role(s) of non-parenchymal cells and cell-cell interactions in the tissue response is not known. • What is meant by persistent AHR activation? What are the required temporal patterns of AHR activation required to induce tumors? • The analysis of early responses relies on associative events since the actual gene expression changes that drive later phenotypic changes not known.

Q, discussion questions; A, AHR case study panel remarks, conclusions and referenced figures.

(2) pre-neoplastic focal tissue changes (i.e. hyperplasia and other histopathological observations).

These three KEs appear to be causal for both hepatic and biliary tumors observed in the cancer bioassay (Figure 2). The panel chose to define the KEs in terms of basic biological processes so they would be applicable to both tumor endpoints. While other KEs may be identified in the future, the panel specifically identified these three during discussion. These KEs can be related to three of the hallmarks of cancer: sustained AHR activation leads to liver toxicity and "sustained

Table 3. AHR case study panel members and affiliations.

	Participant names	Affiliations
Co-chairs	Robert Budinsky, PhD	Dow Chemical Company
	Dieter Schrenk, PhD, MD	University of Kaiserslautern
Rapporteurs	Martin van den Berg, PhD	Utrecht University
	Ted Simon, PhD, DABT	Ted Simon LLC
	Andrew Maier, PhD, CIH, DABT	Toxicology Excellence for Risk Assessment (TERA)
Panel members	Bruce Allen, MS	Allen Consulting
	Melvin Andersen, PhD	The Hamner Institutes for Health Sciences
	Lesa Aylward, MS	Summit Toxicology, LLP
	Amy Brix, PhD, DVM, DACVP	Experimental Pathology Laboratories (EPL)
	Thomas Gasiewicz, PhD	University of Rochester
	Norbert Kaminski, PhD	Michigan State University
	Gary Perdew, PhD	Penn State University
	Thomas Starr, PhD	TBS Associates
	Nigel Walker, PhD	National Institute for Environmental Health Sciences (NIEHS)
Presenters	Melvin Andersen, PhD	The Hamner Institutes for Health Sciences
	Craig Rowlands, PhD	Dow Chemical Company
	Russell Thomas, PhD	The Hamner Institutes for Health Sciences

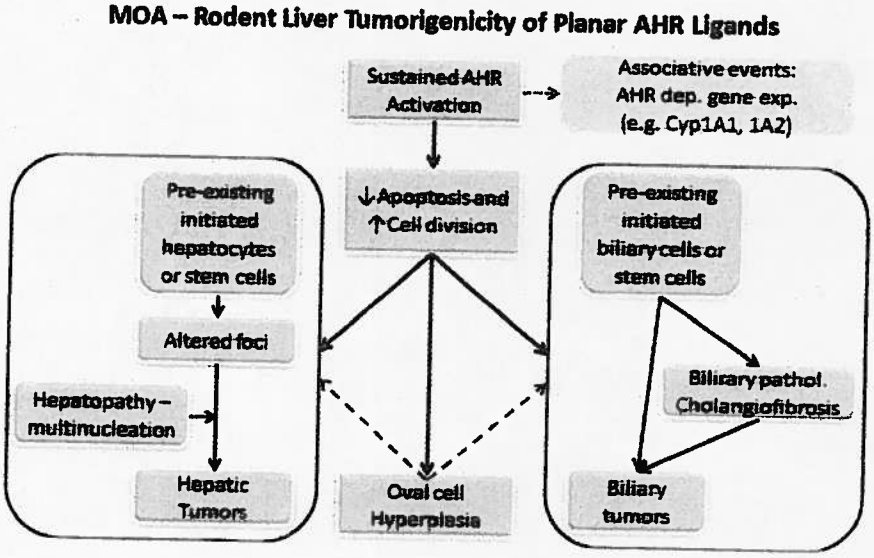

Figure 2. Proposed schematic of the MOA hypotheses developed by the AHR Case Study Panel. Postulated mode of action with key events of rodent liver tumors induced by AHR agonists. Sustained activation of the AHR regulates the transcription of different classes of genes including those involved in cell proliferation and apoptosis. AHR-induced changes in gene transcription occur within altered cells of either a biliary or hepatocellular lineage to expand and promote the eventual development of cholangiolar and hepatocellular adenomas and carcinomas. Inhibition of intrafocal apoptosis also facilitates the survival of initiated cells that would otherwise undergo apoptosis. Central to this tumor promotion scheme is the role of oval cell (stem cell) proliferation with potential impacts on normal differentiation. Histopathological changes noted in the descriptor "hepatopathy", e.g. multinucleated hepatocytes, further contribute to the expansion of pre-neoplastic. At higher-doses, elements of necrosis and regenerative repair may serve to increase cell proliferation.

proliferative signaling" and cell division/decreased apoptosis embody the concept of "resisting cell death" and "evading growth suppressors" (Hanahan & Weinberg, 2011).

In addition to these KEs, the panel also identified AEs and ModFs which play a role in the tumor promotional MOA. More specifically, the results of the expert panel deliberations validated the MOA of TCDD as an AHR agonist that induces both hepatocellular adenomas and cholangiocarcinomas in rats (Andersen et al., in press). A proposed scheme of the MOA hypothesis developed by the panel is shown in Figure 2.

Key events (KEs) and associative events (AEs) as part of the overall MOA

Despite the fact that the definition of a KE includes measurability, obtaining direct measures of KEs is not always possible, especially in humans. In such instances, as in the case of the AHR, AEs provide indirect biomarkers of specific KEs, even though AEs may not be necessary for the apical event to occur. This distinction is made clear in the discussion of apoptosis below. Notwithstanding the argument that all biological measurements of interim biomarkers are indirect, it is nonetheless useful to classify some biological measures as more directly related to the specific KEs than others. Relevant AEs include induction of xenobiotic metabolizing enzymes that are biomarkers indicative of exposure. There are AE's involving changes in AHR-dependent gene transcription, molecular interactions related to the inhibition of intrafocal apoptosis, and possibly other histopathological changes such as multinucleation of hepatocytes that may also precede tumor development (NTP, 2006a,b,c,d).

KE #1 – sustained AHR activation

Sustained AHR activation occurs as a consequence of the continuous presence of activating ligand that binds the AHR resulting in ongoing nuclear translocation and binding of the AHREs. It is generally recognized that the toxic effects of dioxins depend on AHR receptor-mediated modulation of gene transcription (Boutros et al., 2011; Fletcher et al., 2005; Franc et al., 2008; Hayes et al., 2009; Le Vee et al., 2010; Ovando et al., 2006; Sartor et al., 2009; Tijet et al., 2006; Walisser et al., 2005; Weiss et al., 2008). Sustained AHR activation was deemed necessary for the apical effect (tumors) to occur and the acute changes in AHR-activation can be measured by the induction of certain xenobiotic metabolizing enzymes (in the *Ah* gene battery). Sustained AHR activation results in the disruption of normal cellular function and promotes the clonal expansion of altered hepatic foci (Aly & Domenech, 2009; Maronpot et al., 1993; Pitot et al., 1987; Qu & Vondriska, 2009; Teeguarden et al., 1999).

AHR activation by many xenobiotic ligands is normally transient (Mitchell & Elferink, 2009). However, TCDD exposure leads to sustained AHR activation since it is not readily metabolized, binds the AHR with very high affinity, and has a long chemical half-life. When sustained AHR activation occurs in initiated hepatocytes and biliary cells, proliferation is enhanced, and development of altered hepatic foci results. This is a specific mechanism of tumor promotion. Multiple lines of evidence support sustained AHR activation as the central and initial KE in the tumorigenic MOA. These include:

(1) Ligand-dependent modulation of gene transcription is almost entirely eliminated in AHR knockout mice and in the presence of small-interfering RNAs that target the AHR (Le Vee et al., 2010; Tijet et al., 2006). AHR knockout mice are resistant to TCDD-induced toxicity (Fernandez-Salguero et al., 1996; Gonzalez & Fernandez-Salguero, 1998; Gonzalez et al., 1996);
(2) AHR polymorphisms can lower the binding affinity for ligands or can lead to an inability of the transactivation domain to alter gene expression; both types of polymorphisms result in a decreased response to ligand activation (Chapman & Schiller, 1985; Connor & Aylward, 2006; Poland et al., 1994; Viluksela et al., 2000);
(3) AHR ligands with lower binding affinity are less potent with respect to the KEs, AEs, ModFs and tumor promotion (Buchmann et al., 1994; Budinsky et al., 2006, 2010; Schrenk et al., 1994; Van den Berg et al., 2006; Waern et al., 1991);
(4) AHR mutations that prevent nuclear translocation, dimerization with ARNT, and binding to the AHRE protect against dioxin-like chemical toxicity (Bunger et al., 2003, 2008; Gonzalez et al., 1995; Walisser et al., 2005);
(5) In some cases, AHR activity itself may lead to tumorigenicity: a constitutively active AHR increases the incidences of liver and stomach tumors in mice (Andersson et al., 2002; Brunnberg et al., 2006; Moennikes et al., 2004) and
(6) Zonal AHR expression within the liver sinusoid in mice is correlated with hepatotoxicity *in vivo* (Chang et al., 2005).

Negative results in tumor promotion or cancer bioassay studies in knockout animals would provide the most definitive evidence linking AHR activation with tumor promotion; such studies have not yet been conducted. However, liver toxicity is not observed in either AHR knockout mice or dioxin-resistant Han Wistar/Kuopio rats at doses of TCDD that are hepatotoxic in sensitive strains such as Sprague–Dawley rats (Niittynen et al., 2007; Sand et al., 2010). The panel agreed that the overall data support the conclusion that sustained AHR activation is the pivotal and initial KE in the MOA for dioxin-induced rodent hepatocarcinogenesis. Recently, EPA's ToxCast™ program has identified persistent nuclear receptor activation as a non-genotoxic mechanism of rodent liver cancer, a mechanism for tumor promotion, and a likely KE in the MOA for rodent hepatocarcinogenesis, consistent with the finding of the AHR panel (Shah et al., 2011).

There are only a few examples of AHR responses independent of altered gene transcription, e.g. cAMP-related changes in calcium flux (Oesch-Bartlomowicz et al., 2005; Oesch-Bartlomowicz & Oesch, 2009) and direct interaction of the AHR with other proteins. Proteolytic degradation of transcription factors is one means of controlling the transcriptional response to a ligand. Once activated by TCDD or another ligand, the AHR is subject to degradation by ubiquitinylation and binding to the 26S proteasome (Davarinos & Pollenz, 1999; Kinyamu et al., 2005; Nawaz & O'Malley, 2004; Pollenz, 2002; Suzuki & Nohara, 2007). The suggestion has been made that AHR ligands can mediate an inflammatory response by a non-genomic pathway (Matsumura, 2009); however, the available evidence suggests that many genes associated with inflammatory pathways are part of the AHR-inducible battery (Degner et al., 2009; Dong et al., 2010; Yoon et al., 2006).

At present, with the exception of ubiquitinylation as a control mechanism, a role for any potential non-transcriptional events in the proposed tumor promotional MOA cannot be defined. Nevertheless, the evidence supporting the role of AHR activation as a KE in dioxin-induced tumor promotion is extensive and has been accumulating since the discovery of the AHR over 30 years ago. Sustained AHR activation is itself a complex cascade of molecular events, some of which have not been fully characterized. However, for MOA evaluation in the context of health risk assessment, the panel noted that this KE can be adequately represented by the AEs changes in gene transcription and expression of proteins both *in vivo* and *in vitro* (Abraham et al., 2002; Boutros et al., 2011; Budinsky et al., 2010; Drahushuk et al., 1996, 1998, 1999; Haarmann-Stemmann et al., 2007; Kitchin & Woods, 1979; Lambert et al., 2006; Le Vee et al., 2010; Ma & Lu, 2007; NTP, 2006a; Schrenk et al., 1991, 1995; Silkworth et al., 2005, 2008; Toyoshiba et al., 2004; Tritscher et al., 1992; Uno et al., 2009; Vanden-Heuvel et al., 1994; Viluksela et al., 1997, 1998; Walker et al., 1999; Xu et al., 2000; Zhang et al., 2006).

KE #2 – altered focal cell growth/homeostasis

Dioxin-like chemicals are not tumor initiators (Poland & Glover, 1979; Turteltaub et al., 1990; Wassom et al., 1977). Therefore, a tumor promotional action for TCDD must include an increase in cell division or a decrease in apoptosis

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or both (Hanahan & Weinberg, 2011; Roberts et al., 1997). Several lines of evidence support a role for both of these processes as KEs in the tumorigenicity of TCDD and dioxin-like chemicals, and some of the evidence also supports a dose–response threshold for clonal expansion of altered hepatic foci (Pitot et al., 1987; Teeguarden et al., 1999).

Decreased apoptosis

Evidence supports inhibition of apoptosis as part of the tumor promotional MOA of TCDD. Inhibition of apoptosis within altered hepatic foci may be the promotional mechanism underlying tumor progression. Inhibition of apoptosis within the foci results in clonal expansion of phenotypically altered cells that would otherwise undergo programmed cell death (Chopra & Schrenk, 2011).

In vivo *inhibition of apoptosis*

(1) **Dioxin-like chemicals decrease intrafocal apoptosis *in vivo*.** In initiation–promotion experiments where a single administration of diethylnitrosamine produced initiated cells, dioxin-like chemicals were shown to inhibit apoptosis *in vivo* within GSTP-positive (GSTP+) altered hepatic foci. Markers for this effect were identified using both histological identification of apoptotic bodies and immunochemical staining to measure bromodeoxyuridine (BrdU) incorporation in the same tissue slices (Luebeck et al., 2000; Stinchcombe et al., 1995).

(2) **TCDD decreases the occurrence of Caspase 3-positive cells within altered hepatic foci.** Caspases are cysteine-containing proenzymes that become active once a cell begins apoptosis and provide a biomarker for this process (Afford & Randhawa, 2000). Caspase 3 levels, detectable by immunocytochemistry were reduced within GSTP+ foci in rats undergoing an initiation-promotion protocol (Eckle et al., 2004).

(3) **TCDD appears to inhibit apoptosis by reducing levels of p53.** TCDD inhibited apoptosis *in vivo* measured by TUNEL-staining in diethylnitrosamine-initiated rats in the liver, although this observation could not be isolated to altered hepatic foci (Paajarvi et al., 2005). In these experiments, TCDD reduced overall levels of p53 and serine15-phosphorylated p53, both key mediators of apoptosis.

In vivo *inhibition of apoptosis*

(1) **TCDD produces *in vitro* inhibition of apoptosis.** Treatments such as UV irradiation or administration of a genotoxicant produce apoptosis. TCDD inhibits apoptosis produced in this way in both human cell lines and rat primary hepatocytes (Ambolet-Camoit et al., 2010; Chopra et al., 2009, 2010; Schwarz et al., 2000). TCDD by itself produced a slight increase in apoptosis in mouse primary hepatocytes. These differences could be due to the low concentration of TCDD used in these experiments or could be a species-specific effect (Chopra & Schrenk, 2011).

(2) **Similar to the *in vivo* circumstance, TCDD inhibits apoptosis *in vitro* via effects on p53.** Other *in vitro* experiments appeared to confirm that inhibition of apoptosis following TCDD exposure was mediated via the AHR through effects on phosphorylation and presumably inactivation of p53 observed in rat primary hepatocytes (Worner & Schrenk, 1996). In addition, the dose–response for p53 phosphorylation was almost identical to that for induction of CYP1A1 measured by EROD, suggesting that p53 phosphorylation occurred via an AHR-induced kinase (Worner & Schrenk, 1996). The involvement of gene expression in TCDD inhibition of apoptosis is supported by the fact that protein biosynthesis is required for apoptosis-related gene induction including *birc3*, *dad1*, *pycard* and *inf* (Chopra et al., 2009, 2010; reviewed in Chopra & Schrenk, 2011).

(3) **TCDD appears to have minimal or no effect on p53-independent apoptosis.** TCDD had no effect on apoptosis mediated via transforming growth factor-β1 (TGF-β1), which signals through a p53-independent pathway (Cain & Freathy, 2001; Liebermann et al., 1995). In addition, no effect was observed with TCDD in mouse hepatocytes treated with either TGF-β1 or bleomycin (Christensen et al., 1998). The lack of effect on the TGF-β1 pathway (Bauman et al., 1995; Hushka & Greenlee, 1995) is consistent with results from rat hepatocytes where TCDD did not inhibit TGF-β1-induced apoptosis (Worner & Schrenk, 1996). However, bleomycin acts by damaging DNA and induces apoptosis at least partially through the activation of the p53-dependent pathway (Chopra & Schrenk, 2011), and, thus, one might expect TCDD to inhibit bleomycin-induced apoptosis.

Indirect evidence of apoptosis inhibition

(1) Sustained AHR activation leads to clonal expansion of altered hepatic foci produced by nitrosamine administration and/or partial hepatectomy. The number of phenotypically altered foci increases but that may be a result of nitrosamine administration; however, the increase in volume fraction of foci within livers is due to both increases in cell division and decreases in apoptosis (Luebeck et al., 2000; Moolgavkar et al., 1996; Teeguarden et al., 1999). Numerous initiation–promotion studies suggest that TCDD inhibits intrafocal apoptosis (Dragan & Schrenk, 2000). Initiation–promotion studies using doses as low as 10 pg/kg day provide evidence suggesting the presence of a threshold dose for alterations in clonal expansion of altered hepatic foci. Teeguarden et al. (1999) observed increases in volume fraction with both dose and time in four types of altered hepatic foci. For example, Figure 3 shows the effects of both dose and time on the increase in volume fraction of one of four types of foci (Teeguarden et al., 1999). Considering all four types of foci, after 1 month a dose-dependent increase was observed in one of the focal types; after 3 months a dose-dependent increase in two of four types of foci occurred and, after 6 months the volume fraction of all four types of foci were increased.

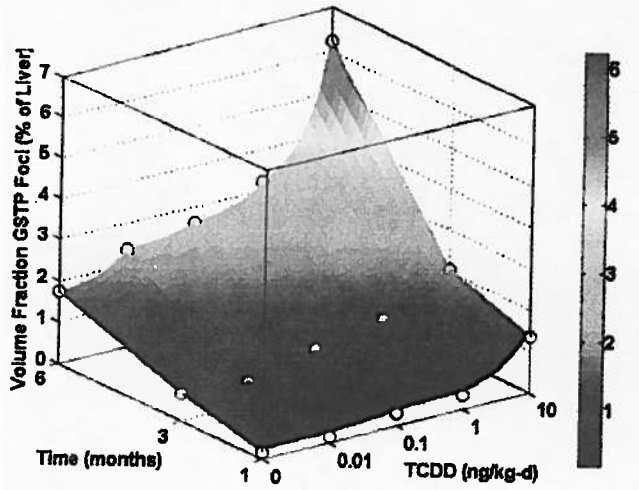

Figure 3. Dose- and time-dependence of the increase in the volume fraction of GSTP foci in an initiation-promotion assay. Data from Teeguarden et al. (1999). The gray circles at 1, 3 and 6 months show data points with the corresponding interpolated surfaces shown in false colors with the color coding shown in the bar to the right. For example, the volume fraction does not get >4% (yellow, orange or red) until after 6 months of treatment with 10 ng/kg day TCDD.

(2) Inhibition of intrafocal apoptosis by TCDD has been linked to expression changes in apoptosis-regulating proteins. These proteins include Mdm2, Tfgb1/4, AGR2 and others (Franc et al., 2008; Paajarvi et al., 2005; Zeytun et al., 2002).

Increased cell proliferation

(1) **Evidence for altered hepatocellular proliferation.** Both increases and decreases in cell division measured by new DNA synthesis have been reported for dioxin-like chemicals. Time-dependent pattern of changes in BrdU labeling indices have been observed in response to TCDD; at early points in time and low doses TCDD produced a decrease in hepatocyte cell division, whereas with longer exposure times and at higher doses TCDD increased hepatocyte cell division. Low-dose exposures to TCDD reduced BrdU labeling in diethylnitrosamine-initiated rats after 30 weeks (3.5 ng/kg day) and 7 months (1 ng/kg day) of administration (Pitot et al., 1987); however, an increase in labeling was observed in initiated rats at the higher doses of 125 ng/kg-TCDD (Maronpot et al., 1993).

(2) Transient decreases in hepatic BrdU labeling have also been observed. Increased Brdu labeling was reported in female rats following a 1-week exposure to 0.3, 30 or 150 ng/g TCDD; however, after 2-week exposures no difference from control was apparent at any dose. Male rats, by comparison, only had significantly decreased hepatic labeling at the end of the first week following exposure to 150 ng/g, which returned to control levels by the end of week 2 (Fox et al., 1993). Similarly, Teeguarden et al. (1999) observed a reduced labeling index in diethylnitrosamine-initiated rats to exposed to either 0.1 or 1 ng/kg day TCDD for 1 month and exposed to 0.1 ng/kg day TCDD for 3 months. At 6 months, the labeling index was not different from control at any TCDD dose.

Increased labeling index over controls was observed in tissues from animals exposed to higher doses *in vivo* (Hailey et al., 2005); after 31 weeks, the labeling index was increased at all doses (from 3 to 100 ng/kg day); however, at 53 weeks, the labeling index was increased only at 46 and 100 ng/kg day. In non-initiated female rats given 125 ng/kg day TCDD, Walker et al. (1998) observed a decrease in labeling after 14 weeks and an increase after 60 weeks, while in diethylnitrosamine-initiated rats, significant increases were observed after 30 and 60 weeks. To summarize, at low doses and early time points, TCDD reduces hepatocyte cell division, but the opposite may occur at high doses and later time points.

(3) **Evidence for altered proliferation in biliary cells.** Increased biliary cell proliferation or a periportal increase in cell division, as indicated by increased BrdU labeling has also been reported following TCDD administration (Bauman et al., 1995; Fox et al., 1993; Maronpot et al., 1993). Consistent with an increase in cell proliferation, rats initiated with diethylnitrosamine and administered 125 ng/kg day TCDD for 30 weeks showed a large increase in expression of transforming growth factor alpha (TGF-α), a growth factor with known tumor-promoting effects in mouse liver (Tamano et al., 1994; Tritscher et al., 1995). In areas of the bile duct close to oval cells and/or differentiating liver stem cells that will become cholangiocytes or hepatocytes, increases in TGF-α could contribute to oval cell and hepatocellular proliferation. At higher doses, necrosis, inflammation and other cytotoxic responses can contribute to the potential for regenerative repair (Hailey et al., 2005; Hemming et al., 1995; Mantovani et al., 2008). Proliferation due to direct AHR activation or induced by regenerative repair likely begins with biliary epithelial cells and/or oval cells. Oval cells in the periportal region and possibly biliary epithelial cells likely function as a source of replacement of hepatocytes after liver injury (Paku et al., 2001; Sahin et al., 2008; Tanaka et al., 2010; Wang et al., 2004). Dose-related changes were observed in bile duct hyperplasia with similar dose–response characteristics for hepatopathy[5] that include bile duct and oval cell hyperplasia as well as other toxic responses in the recent NTP bioassays (Hailey et al., 2005). These

[5]Minimal (grade 1) toxic hepatopathy was diagnosed when additional changes indicative of a toxic effect, usually a slight degree of bile duct and/or oval cell hyperplasia or a few large prominent altered hepatocellular foci, and occasionally a small focus of cholangiofibrosis were present. Mild (grade 2) toxic hepatopathy was characterized by the presence of multiple toxic changes, all of which were of minimal to mild severity. In addition, multiple prominent altered hepatocellular foci (usually mixed cell foci) and an occasional focus of nodular hyperplasia were sometimes present. Moderate (grade 3) toxic hepatopathy was diagnosed when most or all the spectrum of toxic changes were present, with some degree of distortion of the normal liver structure caused by prominent altered hepatocellular foci, nodular hyperplasia and cholangiofibrosis. Marked (grade 4) toxic hepatopathy was diagnosed when severe toxic changes were present with pronounced distortion of the liver architecture. Livers with marked toxic hepatopathy often had a multinodular appearance due to the presence of numerous large foci of nodular hyperplasia that replaced much of the liver parenchyma (Hailey et al., 2005).

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data support the conclusion that chronic administration of TCDD with resulting sustained AHR activation, and the occurrence of hepatopathy (indicative of the constellation of histopathology changes that precedes tumors) are required for proliferation to occur. This increased cell proliferation is a late stage event consistent with the late onset of hepatic and bile duct tumors.

Effects on proliferation are time-dependent

Timing is key when characterizing the cell proliferation response to AHR activation; early AHR activation results in cell cycle arrest in normal hepatocytes, whereas regenerative repair and hyperplasia both occur well after sustained AHR activation has begun, and at a time when significant hepatic histopathology is evident. The more immediate cell cycle delay caused by acute AHR activation is discussed under ModFs below. Inhibition of intrafocal apoptosis, discussed earlier, is one factor that results in increased cell proliferation. Bile duct fibrosis is an indication of a mitogenic stimuli in the acinar region where oval cells reside (Hailey et al., 2005). Sustained or chronic AHR activation at higher TCDD dosages produces cytotoxicity, and this can lead to accompanying regenerative repair. Any non-neoplastic histopathological changes are described as "toxic hepatopathy", a generalized index of severity for non-neoplastic liver changes (Hailey et al., 2005; NTP, 2006a,b,c,d).

In summary, intrafocal inhibition of apoptosis results in increased cell proliferation and tumor promotion. At higher TCDD dosages and later times, increases in cell division may also be directly stimulated, and/or increased by cytotoxicity-driven regenerative repair.

KE #3 – pre-neoplastic focal tissue changes (histopathological evidence)

Pre-neoplastic changes noted in histopathological alterations are a significant KE underlying sustained AHR-mediated tumor promotion in female rats (Goodman & Sauer, 1992; Hailey et al., 2005). Liver toxicity associated with tumor development is also observed in the mouse and occurs even earlier than it does in TCDD-treated rats (Chang et al., 2005; NTP, 1982). In the case of the AHR, a number of AEs provide opportunities for quantitative dose–response modeling as a path to understanding the MOA (Figure 4). Morphological AEs reflective of hepatopathy include histological evidence of steatosis (fatty change), mitochondrial injury, necrosis, fibrosis and porphyria (increased pigmentation) (Boverhof et al., 2005; Chang et al., 2005; Hailey et al., 2005; Jones & Greig, 1975; NTP, 2006a,b,c,d; Shertzer et al., 2006; Walker et al., 2006). Cell division (Figure 4) and necrosis, which together contribute to regenerative repair, appear to be late events associated with high doses, indicating they are likely to be non-linear phenomena. It should be noted, however, that hyperplastic nodules associated with regeneration cannot be morphologically distinguished from a hyperplastic nodule of another pathogenesis. Taken together the morphological changes suggest regeneration is a significant contributor to the proliferative response in animals with toxic hepatopathy. In the NTP TCDD bioassay (NTP, 2006a), proliferative lesions were observed in animals that lack toxic hepatopathy, suggesting that stimuli other than degeneration and necrosis may contribute to proliferative lesions. Since the NTP studies did not report changes related to hepatic zonation, the possibility exists that specific hepatic regions are impacted prior to the appearance of morphologic changes. In the livers of rats given dioxin-like chemicals, AHR activation and liver toxicity first appear in centrilobular regions and later extend throughout the liver (Goodman & Sauer, 1992; NTP, 2006a,b,c,d). These results, however, do not preclude the possibility of early histopathological impacts of AHR activation in other hepatic zones. Both the ubiquity of toxic hepatopathy and the regenerative nature of the liver itself support unrestrained cell division as a KE in the tumor promotional MOA of dioxin. Histological and immunocytochemical evidence supports a causal relationship between AHR activation and hepatotoxicity (Chang et al., 2005). Therefore, the KEs of cell proliferation (KE #2) and the non-neoplastic histopathology for this KE (#3) may provide the link between sustained AHR activation and the tumor occurrence.

Modulating factors

Modulating factors are conditions or responses that interact with KEs and can alter the dose–response and time-dependent relationships of KEs and thus, the apical event in the MOA. ModFs cannot, however, alter the necessity of the occurrence of KEs for the apical event to occur. ModFs themselves may be AHR-dependent or -independent. The effect of various ModFs could be either to enhance or to inhibit tumor promotion. The specific ModFs considered by the panel included estradiol co-promotion, oxidative stress, inhibition of cell communication, mitoinhibition and other cell cycle dysregulation, constitutive activity and endogenous AHR ligands, and impacts of zonation of AHR activation.

Certain AHR-dependent responses appear to be cancer-protective (Marlowe & Puga, 2005). These may also be considered as potential ModFs and include dioxin-inducible cell cycle arrest, as well as the competing/augmenting role of naturally occurring endogenous and dietary AHR ligands. In the future, the incorporation of quantitative information on ModFs into the MOA could reveal whether integration of the effects could affect the tumor dose–response.

Estrogen and estradiol as modulators of AHR activity

In rats, a female-specific liver tumor response suggests an interaction between AHR activation and estradiol and/or the estrogen receptors. It is known that estrogen itself is capable of causing tumor promotion (Graham et al., 1988; Hiraku et al., 2001; Lucier et al., 1991; Vickers & Lucier, 1996; Vickers et al., 1989; Yager & Yager, 1980). In mice, this interaction is more complex or not present since both males and females are affected. Estradiol metabolism may be one factor since it is known that 4-hydroxyestradiol, the product of CYP1B1 hydroxylation, is associated with oxidative DNA damage. However, 2-hydroxyestradiol, the product of CYP1A hydroxylation is not associated with oxidative DNA damage or DNA adducts. Thus, the ratio of CYP1B1-dependent 4-hydroxylation and CYP1A-mediated 2-hydroxylation of estradiol may ultimately determine if an estrogen-based ModF

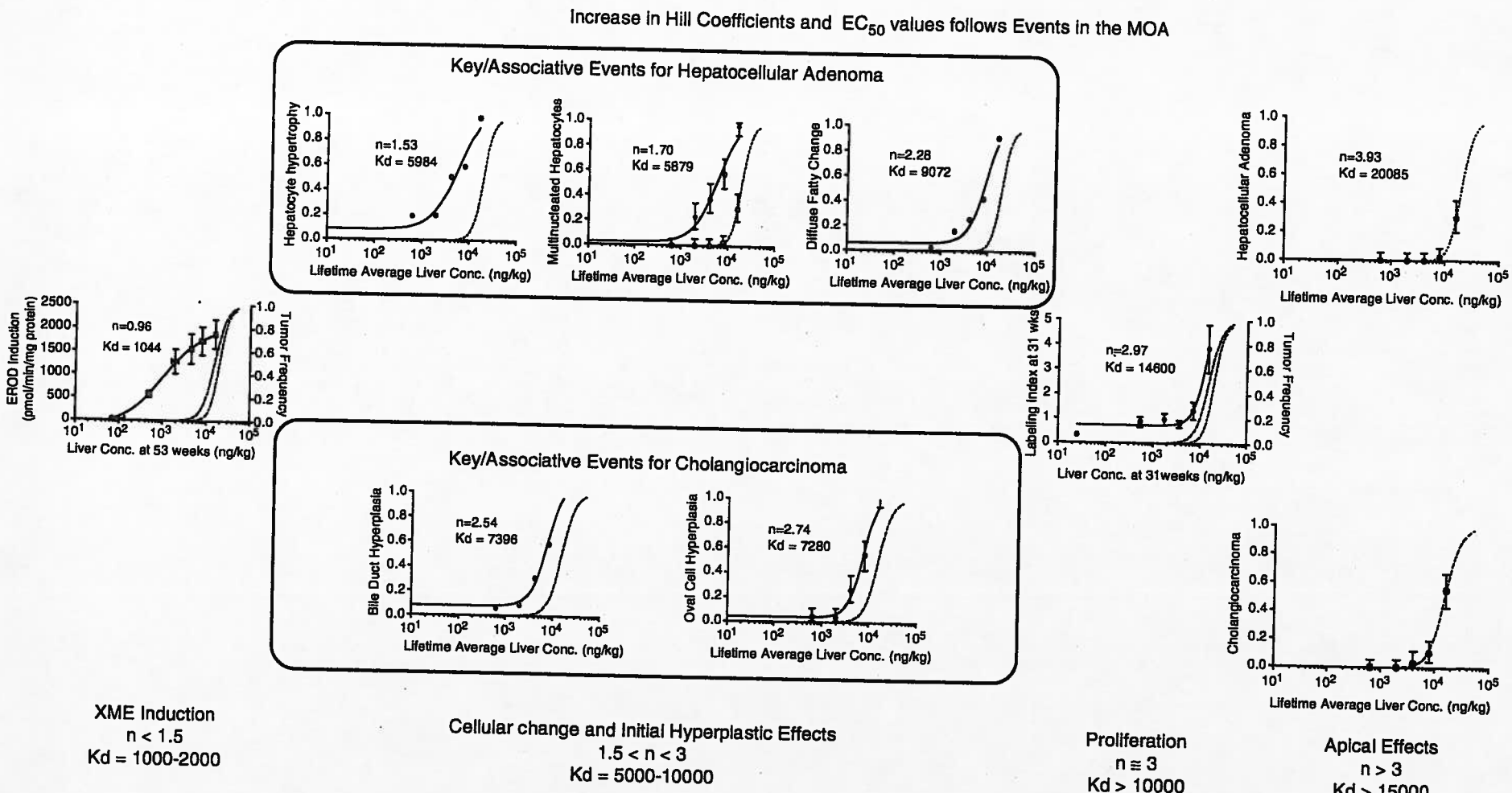

Figure 4. Mode of action of TCDD-induced liver cancer in rats using lifetime average liver concentration (LALC) as the dose (apical endpoint curves (dashed and dotted lines) are overlaid on each non-apical endpoint plot (solid lines) to provide a convenient reference for comparison; for EROD at 53 weeks and labeling index at 31 weeks, a right *y*-axis is included for clarity). The first KE, sustained AHR activation (XME induction; xenobiotic metabolizing enzyme), is measured by dose-dependent induction of EROD, representing the enzymatic activity of CYP1A1. This early low dose–response would likely be linear at low dose because the Hill coefficient (n) is <1.5 (see text for details). As later events in the MOA occur, both the Hill coefficients and the half-maximal concentrations (Kd) increase. Cell proliferation is a seemingly late high-dose event; its Hill coefficient is ~3 and the half-maximal value is >10 000 ng/kg. For the two apical events, hepatocellular adenoma and cholangiocarcinoma, the Hill coefficients are >3 and the half-maximal values are >15 000 ng/kg. This suggests that the apical endpoints are non-linear phenomena and that their occurrence is associated with high doses relative to earlier KEs.

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contributes to TCDD-induced tumor promotion in female rats (Badawi et al., 2000; Cavalieri et al., 2000; Jefcoate et al., 2000; Reichard et al., 2005; Rifkind, 2006; Walker et al., 1999). The evidence suggests, however, that direct genotoxic damage in liver is not likely since estradiol-DNA adducts have not been found *in vivo* (Randerath et al., 1990; Turteltaub et al., 1990). This may contrast with some breast tumors since estrogen-DNA adducts have been discovered in women with breast cancer and have been experimentally identified in TCDD-treated mammary cell lines (Gaikwad, 2008; Lu et al., 2007).

Alternatively, it is plausible that cross-talk between the AHR pathway and the estrogen receptor pathway may be a contributing factor. 17α-ethylestradiol in the absence of TCDD has been shown to act as a liver tumor promoter in rats (Vickers et al., 1989). The AHR is able to recruit the unbound estrogen receptor alpha (ERα) to AHR regulated genes. In addition, some AHR ligands may produce an interaction of the AHR with ERα and modulation of ER signaling (Matthews & Gustafsson, 2006). This may account for some of the differences in cancer response between males and females discussed earlier (Supplemental Figure). However, the explanation for the different pattern of gender responses in mice and rats remains unknown. Overall, estrogen or the estrogen receptor does have a role in the AHR MOA in female rats, although the mechanism(s) for this interaction is not yet defined and the data suggest several possibilities.

Oxidative stress and effects on mitochondria

Oxidative stress is hypothesized to play a role in tumor promotion and cytotoxicity (Goetz & Luch, 2008; Roy et al., 2007). Oxidative stress and the presence of reactive oxygen species (ROS) have been proposed as a possible MOA for TCDD. The basis for this response is ROS generated by disruption of mitochondrial function, the futile cycling of induced CYP1A or CYP1B1 enzyme activity with estradiol quinone formation. Involvement of oxidative stress responses in the MOA is supported by *in vivo* studies using TCDD. Wahba et al. (1989) observed a dose-dependent increase in the production of thiobarbituric acid reactive substances (TBARS) in female Sprague–Dawley rats 7 days after a single gavage dose of between 25 and 100 μg/kg TCDD. It is noteworthy that in rats these are very high TCDD doses that are likely to be acutely fatal. In rats in an initiation promotion protocol, indole-3-carbinol (a high-affinity AHR ligand found in broccoli) produced a dose-dependent increase in the area of altered foci, induction of AHR- and NRF2-mediated gene expression, and evidence of oxidative stress (Shimamoto et al., 2011). In female SD rats, similar dose-dependent increases were observed in ROS-related endpoints measured in liver and brain after administering TCDD, 2,3,4,7,8-PeCDF or PCB-126 for 13 weeks (Hassoun et al., 2000). Since no dose-responsive increase in brain tumor incidence was observed in the NTP (2006) study while ROS-endpoints were dose-dependent over a similar dose range in both liver and brain, it could be argued that the evidence for oxidative stress as necessary for tumor promotion is not sufficient.

An indirect genotoxic MOA has been proposed in which induced CYP1A produces DNA-reactive or ROS-generating estradiol metabolites (Graham et al., 1988; Wyde et al., 2001b), as described in the section on estradiol interactions. Whether sustained AHR activation generates ROS due to estradiol metabolism or other reactions catalyzed by CYP1A or 1B1 is not known. Although oxidative DNA damage has been observed in primary culture rat hepatocytes (Knerr & Schrenk, 2006; Knerr et al., 2006), oxidative DNA adducts, such as 8-Oxo-2′-deoxyguanosine (8-oxo-dG), are not observed in female SD rats treated with dioxin-like chemicals. After 20 weeks of dosing with 100 ng/kg day of TCDD, there was no evidence of an increase in 8-oxo-dG DNA adducts, unless the animals had been initiated with a necrogenic dose of diethylnitrosamine (Wyde et al., 2001a). In addition, an increase in DNA adducts could not be observed in female rats administered 1 and 5 μg/kg week TCDD for 4 weeks (Randerath et al., 1990). Similarly, no dose-dependent increase in DNA adducts was observed in mice given single doses of TCDD between 0 and 500 ng/kg (Turteltaub et al., 1990). Thus, although it is plausible that oxidative stress contributes to the overall toxicity of TCDD, these results suggest that oxidative stress-mediated DNA damage is not a contributing factor necessary for the tumorigenic effects of TCDD, especially at the lower dosages of TCDD that lead to liver tumors.

A second proposed mechanism for oxidative stress generation involves reactions at the level of the mitochondria. Because CYP1A1 and CYP1A2 are localized between the inner and outer mitochondrial membrane, the induction of CYP1A by TCDD may play a role in the generation of mitochondrial-associated ROS (Anandatheerthavarada et al., 1999). In rat primary hepatocytes, there was a dose-dependent increase in ROS generation, hydrogen peroxide formation and the protein carbonyl content of the mitochondria, all measures associated with oxidative stress (Aly & Domenech, 2009). In mice, TCDD also appears to induce oxidative stress characterized by increased mitochondrial respiration, associated ROS production and hyperpolarization of the inner mitochondrial membrane (Shen et al., 2005; Shertzer et al., 2006). The mitochondrial-associated ROS result from disruption of the electron transport chain and oxidative phosphorylation (Senft et al., 2002).

The role of ROS as a ModF for the tumor response may result from mitochondrial oxidative stress and secondary generation of cytotoxicity with sustained regenerative proliferation, rather than as a direct or indirect DNA-reactive MOA. This conclusion is supported by the general absence of positive results in genotoxicity assays with TCDD (Randerath et al., 1990; Turteltaub et al., 1990). Another factor complicating the assessment of a potential role of oxidative stress as a ModF is the activity of TCDD and other AHR ligands as inducers of antioxidant responses. For example, the AHR mediates the induction of anti-oxidant functions and phase II metabolism that may be a protective mechanism in hepatocytes. In mice, 24 h after a single i.p. administration of 50 μg/kg TCDD, there was increased expression of the nuclear transcription factor erythroid-2-related factor 2 (Nrf2, officially Nfe2l2) and its target genes, including NAD(P)H:quinone oxidoreductase 1 (Nqo1). In addition,

there was increased induction of UDP-glucuronosyltransferase 1a6 (Ugt1a6) and glutathione-S-transferase a1 (Gsta1), for which Nrf2 serves as the transcription factor (Yeager et al., 2009). Nrf2 activates its target genes by binding to antioxidant response elements on DNA. In the livers of CYP1A1/1A2/1B1 triple knockout mice, TCDD induces Nqo1, Ugt1a6 and Gsta1 but not the CYPs (Dragin et al., 2008). Other Ugt and Gst isoforms are also induced by TCDD (Boverhof et al., 2005; Buckley & Klaassen, 2009; Knight et al., 2008; Tijet et al., 2006; Wu et al., 2008). These phase II enzymes are generally considered "detoxifying" and thus protective. Similarly, Boverhof et al. (2006) and others have observed gene expression changes in rats consistent with an adaptive response to oxidative stress. TCDD also produces an increase in the transcription of metallothionein, a scavenger of hydroxyl and superoxide radicals that may protect against the oxidative stress produced by TCDD (Nishimura et al., 2001). However, in an initiation–promotion assay using several promoting agents including the nuclear receptor agonist phenobarbital, metallothionein was not histologically associated with GSTP+ foci or proliferating cell nuclear antigen (PCNA), suggesting that oxidative stress was not occurring within altered hepatic foci (Mizukami et al., 2010). Overall the weight of evidence indicates that, although TCDD may produce oxidative stress, it is not a key event in the MOA for tumor promotion by TCDD.

Reduced immune surveillance

TCDD may alter T cell differentiation leading to increased regulatory T cells (Treg cells), which in turn could lead to reduced T cell mediated tumor surveillance (Kerkvliet et al., 2009). This is a plausible mechanism of preneoplastic cell expansion that could allow the TCDD-induced liver tumor cell growth and remains to be explored.

Inhibition of cell communication

Gap junctions are hydrophilic channels connecting the lateral plasma membranes of adjacent cells that allow direct exchange of small cytoplasmic molecules and play an important role in contact inhibition in epithelial cells (Mroue et al., 2011; Rela & Szczupak, 2004). Cell–cell contact is a critical regulator of proliferation, differentiation and motility (Eagle & Levine, 1967). In a number of tissues, loss of cell–cell contact may activate the AHR in the absence of exogenous ligands (Cho et al., 2004), and exogenous ligands may disrupt cell–cell signaling and contact inhibition (Milstone & LaVigne, 1984; Munzel et al., 1996). In primary cultured rat hepatocytes, TCDD down-regulated gap junction intercellular communication in a concentration- and time-dependent manner (Baker et al., 1995). In WB-F344 rat liver stem cells, release from contact inhibition involved activation of the AHR, but did not involve ARNT; thus, AHR activation may affect contact inhibition in a fashion that is different from the classical AHR-ARNT pathway (Weiss et al., 2008). Because the large majority of contact inhibition studies have been performed in oval cell-like WB-F344 cells *in vitro*, the relevance of these results to hepatocytes or to oval cells *in vivo* remains uncertain (Tsao et al., 1984). Nonetheless, these AHR-mediated effects could play a modulatory role in conjunction with the key events in promoting tumor formation.

Other modulating factors altering proliferation

The interactions of proliferation signals among liver cell types are complex and not fully understood; however, growth signaling interactions among cell types have been described. For example, stellate cells in humans and animals can store up to 80% of the vitamin A in the body as retinyl palmitate. During hepatic regeneration, this vitamin A pool is depleted (Pintilie et al., 2010; Senoo et al., 2010; Shmarakov et al., 2010). Liver injury also induces the release of the mitogenic cytokines TGF-α and EGF from stellate cells (Friedman, 2008). In rodents, TCDD also induces retinol loss from hepatic stellate cells (Fattore et al., 2000; Hakansson & Hanberg, 1989; Kelley et al., 2000; Schmidt et al., 2003). Thus, it is possible that sustained AHR activation provides a third type of proliferative stimulus due to the loss of vitamin A and/or the release of cytokines from stellate cells. It should be noted that stellate cells are activated by liver injury and produce collagen, possibly resulting in biliary fibrosis. Interactions between stellate cells and oval cells may thus contribute to the ductular reaction and cholangiocarcinoma (Friedman, 2008).

Kupffer cells are macrophages resident within the liver and respond to liver injury by releasing an array of mediators of inflammation, growth and oxidative stress. Kupffer cells respond to inflammatory stimuli through production of the cytokine TNF-α that has been linked to inhibition of apoptosis (Roberts et al., 2007). TNF-α and IL-1β both mediate inflammation and both were elevated in the livers of rats administered TCDD (Fan et al., 1997). Kupffer cells may not be the only source of TNF-α or other cytokines; this cytokine is produced in both primary rat hepatocytes and WB-344 cells in response to TCDD (Chopra et al., 2009; Umannová et al., 2007). TGF-β is an extracellular cytokine that regulates apoptosis and cell division (Bock & Kohle, 2005). TGF-β activity may be regulated by AHR activation through the latency associated protein (LTBP-1) (Gomez-Duran et al., 2009). In summary, non-parenchmal cells in the liver likely act in a coordinated fashion to produce a response to TCDD-mediated liver injury including production of inflammatory cytokines and growth factors (Kmieć, 2001; Malik et al., 2002; Neuman, 2001). TNF-α, IL-1β and IL-6, the epidermal growth factor (EGF) family, and TGF-β, all appear to be involved in the early responses to TCDD (Haarmann-Stemmann et al., 2009). Kupffer cells not only produce a cytokine signal but also likely coordinate the response in multiple cell types due to their ability to move throughout the liver.

Mitoinhibition and AHR-mediated cell cycle dysregulation may confer a selective growth advantage to initiated cells

Given that some of the KEs in the MOA for TCDD carcinogenesis in rats involve inhibition of apoptosis and increased cell division, inhibition of mitosis might seem somewhat paradoxical. However, it has been suggested that somewhere in the dose-time continuum of chronic AHR activation by TCDD, a switch between tumor suppression and

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tumor promotion occurs when proliferation barriers are overcome and responsiveness to environmental signals is compromised (Puga et al., 2002). This switch from growth suppression to proliferation might be characterized as "evading growth suppressors", one of the hallmarks of cancer (Hanahan & Weinberg, 2011). Therefore, it is possible that at some point in the progression to cancer, this switch results in a phenotypical remodeling of liver cells towards pre-cancerous lesions. This response includes inhibition of apoptosis and enhanced cell division, rather than cell cycle arrest (Bauman et al., 1995; Fox et al., 1993; Marlowe & Puga, 2005; Mitchell et al., 2006; Puga et al., 2002).

Tumor promotion can also be influenced by the ability of AHR activation to induce cell cycle arrest in normal hepatocytes (Bauman et al., 1995; Huang & Elferink, 2005; Hushka & Greenlee, 1995; Mitchell et al., 2006). TCDD exposure causes cell cycle arrest at either the G0/G1 transition or G2/M transition, resulting in diminished capacity for DNA replication and inhibition of cell division (Puga et al., 2002). TCDD inhibits DNA synthesis in partially hepatectomized rat liver and in rat primary hepatocytes through interactions with TGF-β and EGF (Bauman et al., 1995; Hushka & Greenlee, 1995). AHR activation may also arrest cell cycle progression through responses (e.g. Nrf2 induction) and interactions with the retinoblastoma protein (RB) as well as induction of p27 and the consequent inhibition of cyclin-dependent kinase 2 (CDK2) (Ge & Elferink, 1998; Kohle & Bock, 2006; Kolluri et al., 1999; Puga et al., 2000; Weiss et al., 1996; Yeager et al., 2009). TCDD also causes phosphorylation of p53 and presumably cell cycle arrest in a concentration-dependent fashion that is similar to CYP1A induction (Schrenk et al., 2004). In partially hepatectomized mice, TCDD suppressed hepatocyte replication (Mitchell et al., 2006). In its basal state in the absence of a xenobiotic ligand, the AHR may function as a tumor suppressor by regulation of cell division, expression of inflammatory cytokines and DNA repair (Fan et al., 2010). However, in contrast to normal hepatocytes, initiated cells within altered hepatic foci proliferate and the volume fraction of these foci increases with TCDD-induced sustained AHR activation.

Overall, one can speculate that the combination of cell cycle arrest in hepatocytes and inhibition of apoptosis within foci provides a selective advantage for altered cells. One can further speculate that tumor promotion following sustained AHR activation may actually represent a tipping of the balance between forces that inhibit and forces that promote cell division.

Endogenous AHR ligands and constitutive activity

The endogenous activity of the AHR may play an undefined role in the MOA and key event pathway. It is well known that a number of naturally occurring dietary and endogenously formed AHR ligands exist. These naturally occurring ligands may explain why AHR activity measured in human blood is orders of magnitude higher than AHR activity from anthropogenic chlorinated chemicals like TCDD, present in the low part per trillion (ng/L) range (Connor et al., 2008; Schecter et al., 1998). Peterson et al. (2009) reported measurable increases in CYP1A2 induction due to consumption of cruciferous and apiaceous vegetables comparable to CYP1A2 induction due to cigarette smoking, and about a 10-fold lower induction than measured in humans poisoned with TCDD (Abraham et al., 2002). It is currently unknown if naturally-occurring AHR ligands are also present in the blood of rats, but recent work indicates that tryptophan and indole derivatives are ligands capable of activating the AHR *in vitro;* therefore, they could also be expected to activate the AHR *in vivo* (Chiaro et al., 2007; Mukai & Tischkau, 2007; Song et al., 2002; Wincent et al., 2009). Whether or not naturally occurring AHR ligands act as ModFs during TCDD-induced tumor promotion is unknown. The interaction between TCDD and naturally occurring AHR ligands, either in terms of ligand binding or transcriptional network interactions, remains to be investigated with respect to modification of the dose–response relationship (Fan et al., 2010; Sartor et al., 2009).

Hepatic zonation and AHR activation

Aryl hydrocarbon receptor activation is a zonal event that first occurs in centrilobular (zone 3) hepatocytes in conjunction with the onset of hepatotoxicity in mice (i.e. edema, necrosis, steatosis and inflammation). This zonal phenomenon has been modeled in order to evaluate the dose-dependency and non-linearities of AHR activation (Andersen et al., 1997; Chang et al., 2005; Christoffels et al., 1999; Lindros et al., 1997, 1998; Oinonen & Lindros, 1998; Santostefano et al., 1999; Sheikh-Bahaei et al., 2010; Tritscher et al., 1992; Walker et al., 1998; Wambaugh & Shah, 2010). As the dose of TCDD increases, the extent of AHR activation radiates outward through the acinus to impact zones 2 and 1 nearer to the periportal region of the lobule (Bars & Elcombe, 1991). Thus, the lower dosages of TCDD appear to first involve the older, polyploidal hepatocytes that are programmed for senescence (Gupta, 2000). This zonal regulation of several AHR-controlled genes has been reported to involve the Wnt/β catenin signaling pathway and the EGF/Ras/MAPK cascade (Braeuning, 2009; Braeuning & Schwarz, 2010; Braeuning et al., 2009; Giera et al., 2010). Zonal activation may explain tumor promotion of spontaneously developing foci that develop among the older, polyploidal hepatocytes located in the centrilobular region.

However, it remains unclear whether tumors arise in diploid or polyploid cells. ATPase deficient foci in phenobarbital-treated rats are diploid or tetraploid (Sarafoff et al., 1986). Conversely, Gil et al. (1988) showed that in rats treated with nitrosomorpholine and aflatoxin B1, the small cell hyperbasophilic foci were predominantly diploid whereas the ploidy of foci of clear cells, mixed cells or large basophilic cells was the same as the surrounding parenchyma. In primary cultures of hepatocytes from rats undergoing an initiation–promotion protocol, ~80% of the GGT+ cells were diploid and a much lower fraction of GGT-hepatocytes were diploid (Sargent et al., 1989). Finally, the ploidy distribution within GGT+ foci could be altered by dietary choline (Wang et al., 1990).

Zonal AHR activation, however, does not directly explain oval cell proliferation. Oval cells reside periportally in the

canals of Hering, and, in contrast to enzyme induction or hepatotoxicity, oval cell hyperplasia is a high dose, late occurrence phenomena (Hailey et al., 2005). Increased BrdU labeling has been observed within the periportal region following TCDD-induced tumor promotion, though labeling was not specifically linked to oval cells (Maronpot et al., 1993). Oval cell proliferation appears to be secondary to mitogenic signals carried by the bile from centrilobular cells to the periportal region. For example, the AHR has been shown to regulate expression of the potent mitogen epiregulin and IL6 in a DRE dependent manner (DiNatale et al., 2010; Patel et al., 2006). Thus, one possibility is that AHR ligands upregulate mitogen expression and response directly in the oval cells. Both differentiation and neoplastic transformation of bipotential oval cells in the canals of Hering appear to be highly modulated by the local microenvironment. A number of cell types also reside in the oval cell niche including stellate cells, hepatocytes, cholangiocytes, Kupffer cells, fibroblasts and inflammatory cells. These non-parenchymal cells may play a role in oval cell proliferation caused by AHR activation within the acinus (Gaudio et al., 2009). Hence, the oval cells are subject to an ever-changing mélange of autocrine and paracrine signals (Alison et al., 2009; Apte et al., 2008; Yang et al., 2008). An interesting area for future research, that is reflected in the dashed lines connecting oval cell hyperplasia to both the hepatocellular and cholangiolar tumor types shown in Figure 2, is the question of how sustained AHR activation influences stem cell differentiation and stellate cell involvement with stem cells (oval cells in rats). Finally, AHR-induced mitoinhibition among centrilobular hepatocytes may stimulate oval cell division to replace parenchymal cells, normally a function of hepatocyte replication, which may act as a tumor promotional force. In summary, zonal activation has been described as a non-linear phenomenon that can explain some of the hepatic cell responses to TCDD, but it is still undetermined how zonal activation affects or impacts stem cell proliferation in the periportal region.

Hill's modified considerations for causality as they relate to the hypothesized mode of action

The *Guidelines for Carcinogen Risk Assessment* (USEPA, 2005) recommend that experimental support for a hypothesized MOA be discussed from several viewpoints using the Bradford-Hill causal association analysis (Sonich-Mullin et al., 2001). These considerations are also part of the human relevance-MOA framework. Therefore we present below an evaluation of causality for the proposed MOA and KE for AHR-mediated liver tumors described in the context of the Hill considerations and MOA framework components.

Strength, consistency and specificity of association

Evidence for these Hill considerations is based on statistical significance and biological importance for KEs and tumor formation. For example, the relationships between decreased intrafocal apoptosis with increased cell division, tumor formation and sustained AHR activation are statistically significant, thereby giving strength to intrafocal apoptosis as a KE in the MOA (Buchmann et al., 1994). Consistency of KEs in the MOA is supported by multiple lines of evidence that sustained AHR activation is the pivotal event in tumorigenic MOA of TCDD and other AHR ligands. As detailed in the *Key Events* section, loss of AHR activity through mutation, polymorphism or knockdown is associated with a loss of ligand-mediated gene transcription and resistance to TCDD-induced toxicity. Conversely, constitutive AHR activity and sinusoidal AHR expression in mice increase the incidence of tumors and hepatotoxicity, respectively (Chopra & Schrenk, 2011). The array of studies on TCDD consistently demonstrates the formation of preneoplastic hepatic lesions following activation of the AHR with TCDD or related AHR ligands. Specificity of association is supported by experiments showing that changes in the AHR, including mutations, polymorphisms and factors that influence ligand-dependent AHR activation, directly and measurably impact KEs and AEs.

Dose–response concordance

This criterion considers the correlation of dose with KEs and tumor formation, as well as the observation that early KEs result from doses at or below those that cause the apical event. As detailed in Figure 4 and Table 4, the proposed KEs in the MOA meet the Hill considerations for causality, including dose–response concordance. As discussed in detail in Section F below, the more apical the event, the steeper the dose–response slope, as evidenced by increasing Hill coefficients (Figure 4).

Temporal relationship

Causality requires the consideration of the temporal order of KEs leading to the formation of the apical event. The dose-temporality concordance table (Table 4) provides a review of data from independent studies. The information supports the hypothesized MOA because KEs occur in the same order (i.e. KE1 before KE2, then KE3) and all the KEs are listed as occurring well in advance of tumor development. It is also possible that KEs occur parallel to each other, but as in any process, an orderly and logical sequence of the KEs must exist relative to the biological progression of tumor promotion to the apical endpoint.

Biological plausibility and coherence

To establish causality, it must be shown that the hypothesized MOA is consistent with current scientific knowledge for the agent and the biology for development of the apical outcome. The MOA is biologically plausible because TCDD has been shown to cause sustained AHR activation, altered cell growth, preneoplastic changes and tumors in rodents. The hypothesized AHR MOA is supported by the KEs and AEs, consistent with the biology of carcinogenesis and the events of tumor promotion (Dietrich & Kaina, 2010; Gasiewicz et al., 2008; Nebert et al., 1993; Roberts & Whitelaw, 1999). Increased cell proliferation correlates with a number of underlying AEs that have been identified for sustained AHR activation and are strongly associated with the biology of carcinogenesis.

- The hypothesized MOA is also coherent with the current scientific understanding for tumor development.

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Table 4. Application of the dose and temporal concordance Hill considerations for key events in rodents treated with TCDD.

		Temporal														
		Key Event 1	Key Event 2	Key Event 3				Key Event 4						Tumors	
		(Immediate)	(Days to weeks)	(Months)				(Months)							
		AHR Activation/ Transcrip. (XME)	↓ Apoptosis	Proliferation/Hyperplasia				Toxicity							
				AHF Vol.	BrdU LI	Bile duct (BDH)	Oval Cell (OCH)	Multi-nucleate Hepatocytes (MNH) (weeks)			Diffuse Fatty (DFC) (weeks)			Hepatic Adenoma	Cholangio-carcinoma
Dose	Dose ALC (ng/kg)							14	31	53	14	31	53		
	<100		+												
	100–1000	++++	+	+	+										
	1000–2000	++++	+	++	+	+									
	2000–5000	++++	+		+	+									
	5000–10 000	++++		++	+	++			+	+			+	+	+
	>10 000	++++	++	+++	++	++	+	+	++	+	+	+	+	+	+

ALC, average liver concentration; AHF Vol., altered hepatic foci volume; LI, labeling index; XME, xenobiotic metabolizing enzyme.

The hallmarks of cancer constitute an organizing rationale for the complex biologic events of neoplasia (Hanahan & Weinberg, 2011). Through activation of the AHR, dioxin-like chemicals initiate a multistep process whereby some cells acquire the traits that enable them to become neoplastic and ultimately malignant. Although not all the evidence is within the context of the liver or liver tumors, the overall coherence of data with each hallmark contributes to the strength of the hypothesized MOA. This is particularly true since the intent of the evaluation is to exclude human relevance of the MOA, not try to support relevance. Hence, when additional data that is directly applicable to the MOA in humans is available, should be considered. *Sustaining*

- *Proliferative Signaling:* Sustained AHR activation promotes proliferation of hepatocytes and biliary cells, and provides a proliferative stimulus thorough pathways that include growth factor production (e.g. TGF-α), regenerative repair and stellate cell activation.
- *Evading Growth Suppressors:* When proliferation barriers are overcome and responsiveness to environmental signals is compromised, a switch between tumor suppression and tumor promotion occurs. This switch occurs slowly as individual cells acquire sufficient mutations to become initiated and clonally expand into altered foci. Sustained AHR activation promotes clonal expansion of altered hepatic foci and down regulation of gap junction communication, thereby disrupting cell–cell signaling and contact inhibition.
- *Resisting Cell Death*: Sustained AHR activation by TCDD and dioxin-like chemicals inhibits apoptosis within altered hepatic foci. This enables clonal expansion of altered cells that would otherwise undergo programmed cell death; hence, inhibition of apoptosis is a promotional mechanism that may underlie tumor progression. An early response to TCDD-mediated inflammatory hepatotoxicity, increased levels of the cytokines TNF-α and TGF-β have been linked to inhibition of apoptosis.
- *Enabling Replicative Immortality*: Excessive and persistent self renewal signals are one of the key events in the early stages of liver of carcinogenesis (Wicha et al., 2006). Oval cells, which are a liver-specific stem cell, are thought to be normally quiescent but with tremendous replicative potential (Darwiche & Petersen, 2010). TCDD and dioxin-like chemicals promote oval cell proliferation and hyperplasia through regenerative repair, growth factor production and decreased contact inhibition (Dietrich & Kaina, 2010; Faust et al., 2013). Telomerase expression is a key event for replicative immortality. In human choriocarcinoma cells, TCDD alone or in combination with 17-beta estradiol (E2) increases telomerase activity and the expression of the human telomerase catalytic subunit (hTERT) (Sarkar et al., 2006).
- *Inducing Angiogenesis:* The development of new blood vessels from preexisting vessels is required for tumor growth. AHR is known to contribute to vascular homeostasis and is required for tumor angiogenesis (Roman et al., 2009). Loss of the AHR or treatments with

antagonists, such as resveratrol, reportedly inhibit tumor angiogenesis by interfering with VEGF expression and HIF1A (Hypoxia-inducible factor 1-alpha) accumulation (Kundu & Surh, 2008; Zhang et al., 2006). Loss of AHR enhances the expression of HIF1A, ARNT and VEGF in response to hypoxia (Ichihara et al., 2009). Conversely, AHR activation increases VEGF and promotes vascularization of mouse retina (Takeuchi et al., 2009);

- *Activating Invasion and Metastasis*: There is evidence that AHR activation induces expression of genes that contribute to tissue invasion and metastasis. Activation of AHR/ARNT by TCDD increases expression of genes that facilitate invasion of transformed melanoma cells (Ishida et al., 2010; Villano et al., 2006) and anchorage-independent growth of lung adenocarcinoma cells (Chang et al., 2007). Similarly nuclear localization of AHR in human urothelial tumors correlates with pathological tumor stage, histological grade, tissue invasion and poor prognosis (Ishida et al., 2010).
- *Reprogramming Energy Metabolism:* Metabolic wasting is one of the most consistent toxicologic manifestations observed following exposure to dioxin and dioxin-like chemicals, and includes bodyweight loss often accompanied by hypophagia, hyperlipidemia and hypoinsulinemia (Enan et al., 1992; Seefeld et al., 1984). Metabolic changes accompanying TCDD exposure are associated with perturbation of glycolytic and gluconeogenic pathways in mice and choline metabolism in rats (Forgacs et al., 2012). In murine hepatoma cells, TCDD promotes hyperpolarization of the mitochondrial inner membrane via its interaction with ATP5α1, a protein known to play a role in tumor progression and a glycolytic change (Tappenden et al., 2011).

Human relevance and mode of action

The question of human relevance for TCDD-promoted liver tumors was evaluated by applying the IPCS framework and case studies (Boobis et al., 2006, 2009; Cohen et al., 2003, 2004; Dellarco & Baetcke, 2005; Holsapple et al., 2006; Meek, 2008; Seed et al., 2005). As presented earlier, the KEs within the MOA for liver cancer include:

- sustained AHR activation;
- altered cell growth/homeostasis and
- pre-neoplastic focal tissue changes.

In this section, each of these KEs will be considered regarding the potential relevance to humans. The dose–response species concordance table (Table 5) also presents this information in a condensed fashion.

KE #1 – sustained AHR activation

The human AHR binding affinity can be up to an order of magnitude less than that in rodents, and is reflected in the largely negative epidemiological results from highly exposed populations, such as workers and Seveso residents (Akhtar et al., 2004; Boffetta et al., 2011; Cheng et al., 2006; Collins et al., 2009a,b; Connor & Aylward, 2006; Consonni et al., 2008; Fingerhut et al., 1991; Kogevinas et al., 1997; Mannetje et al., 2005; McBride et al., 2009; Ott & Zober, 1996; Pesatori et al., 2009; Steenland et al., 1999). Evidence of AHR activation in humans is available in measurements of CYP1A1 mRNA and CYP1A2 activity (caffeine metabolism), in lesional skin from chloracne patients or in primary human hepatocytes. These findings demonstrate the ability of humans to respond to dioxins but at dosages higher than those that elicit an equivalent response in rodents (Abraham et al., 2002; Kim et al., 2009; Lambert et al., 2006; NTP, 2006a,b,c,d; Schrenk et al., 1995; Silkworth et al., 2005; Xu et al., 2000). The relative responsiveness of human and rodent AHRs has been confirmed using humanized mice with hepatocyte-specific expression of the human AHR. In primary hepatocytes isolated from mice expressing either wild-type or human AHR, the human AHR is much less responsive to low dioxin concentrations ($\leq$1 nM) than it is mouse homolog (Flaveny et al., 2009, 2010). AHR activation has also been reported in humans exposed to TCDD (Figure 5a and b) at blood concentrations of >1000 ng/L (Abraham et al., 2002; Guzelian et al., 2006). Studies in human primary cell lines show dioxin-mediated changes in gene transcription that occur through the AHR, but again, these gene expression changes require higher concentrations of TCDD than those in rodents (Budinsky et al., 2010; Haarmann-Stemmann et al., 2007; Kim et al., 2009; Schrenk et al., 1995; Uno et al., 2009; Westerink et al., 2008; Zhang et al., 2006). Hence, the evidence indicates that sustained AHR activation is both possible and can be shown to occur in humans.

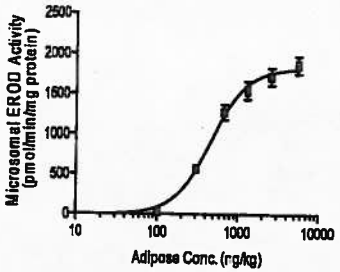
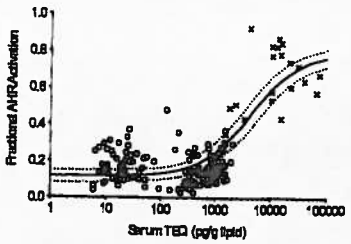
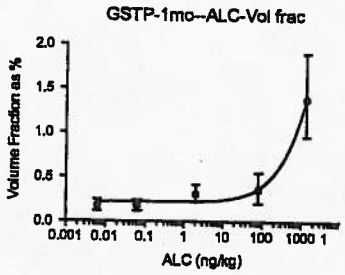
KE #2 – altered cell growth/homeostasis

Beyond simple CYP1A induction and gene expression measured in humans, evidence linking AHR activation with tumor promotion-related KEs in humans is limited. It has been reported that AHR activation produces inhibition of apoptosis in human Huh-7 hepatoma cells (Chopra & Schrenk, 2011). Although data relating AHR activation to liver stem cell proliferation in humans does not yet exist, it is known that a proliferative ductular reaction likely corresponds to oval cell hyperplasia, and that this occurs in alcoholic liver disease and viral hepatitis in humans (Roskams et al., 2004; Sell & Leffert, 2008; Theise et al., 1999).

A potential common biochemical feature of all the above effects on growth may be interference of the AHR with Wnt/β-catenin signaling. This signaling pathways acts in cell–cell adhesion, tissue regeneration and maintenance of liver zonation along the sinusoid. β-catenin acts as a coactivator for many nuclear receptors, including the glucocorticoid receptor, the estrogen receptor and the thyroid hormone receptor. In addition, the AHR and different Wnt protein family members modify the role of β-catenin (Mulholland et al., 2005). AHR modulation of the Wnt/β-catenin pathway may suppress intestinal carcinogenesis in mice (Kawajiri et al., 2009). In humans, the Wnt/β-catenin signaling may be involved in liver cancer; however, the details of this involvement remain to be determined (Behari, 2010).

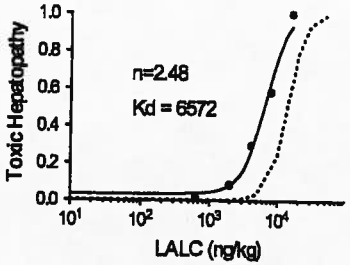
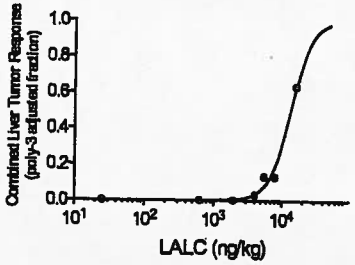
TCDD produces a number of effects related to growth and differentiation in human keratinocytes (Akintobi et al., 2007; Du et al., 2006; Geusau et al., 2005; Loertscher et al., 2001). Many of these are likely related to the development of chloracne and may be mediated by relieving epidermal growth factor receptor (EGFR)-mediated transcription

Table 5. Dose–response concordance table.

Event or factor	Qualitative concordance			Quantitative concordance and quantitative dose–response		
	Animals	Humans	Concordance	Strength	Animals	Humans
KE #1 – Sustained AHR Activation	Occurs in animals *in vivo*	Also occurs in humans *in vivo*	Humans are less sensitive than animals	–+–	 EROD Activity versus Adipose [TCDD] (ng/kg) (NTP, 2006)	 AHR Activation as Caffeine Breath Test versus Lipid-based Serum TEQ
KE #2 Changes in Cell Growth and Homeostasis	Occurs in animals *in vivo*	Has not been observed in humans even at high doses	Unlikely to occur in humans	–		Not observed

(*continued*)

Table 5. Continued

Event or factor	Qualitative concordance			Quantitative concordance and quantitative dose–response		
	Animals	Humans	Concordance	Strength	Animals	Humans
KE #3 – Pre-neoplastic changes	Known to occur in animals *in vivo*	Has not been observed in humans even at high doses	Unlikely to occur in humans	–	Vol. Frac. Of GSTP+ Foci versus Avg. [TCDD] in liver (Teeguarden et al., 1999)  Incidence of Toxic Hepatopathy versus [TCDD] in liver (NTP, 2006)	Not observed
Apical Event – Liver Tumors	Occurs in animals *in vivo*	Has not been observed in humans even at high doses	Unlikely to occur in humans	–	 Combined tumor response versus [TCDD] in liver (NTP, 2006)	Not observed

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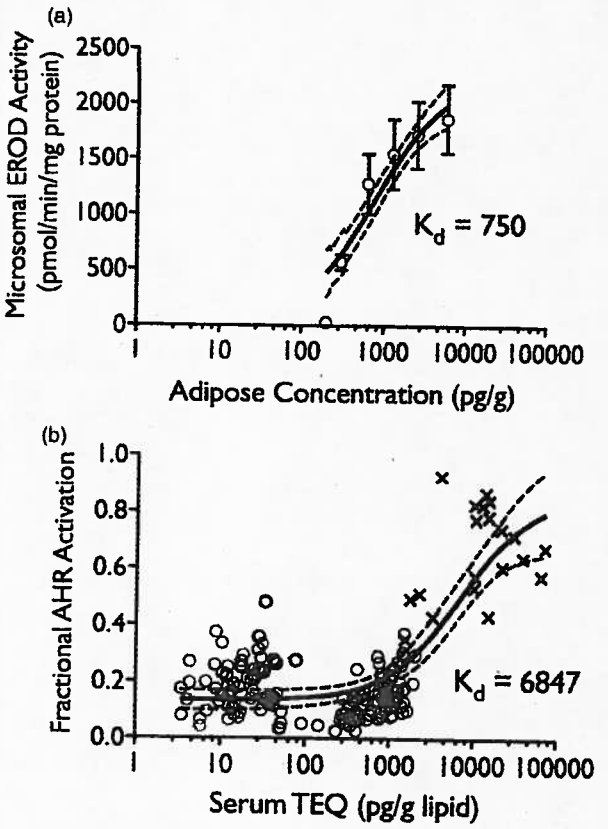

Figure 5. (a and b) Comparison of AHR activation in rats and humans. The upper panel shows EROD activity versus adipose concentration in rats after 53 weeks of TCDD administration (NTP, 2006a,b,c,d). The lower panel shows estimates of fractional AHR activation developed from *in vivo* measurements of caffeine metabolism and CYP1A1 mRNA expression of lesional skin from chloracne patients (Abraham et al., 2002; Coenraads et al., 1999; Lambert et al., 2006; Tang et al., 2008). The human AHR is 10-fold less sensitive to dioxin than the rodent receptor, requiring a 10-fold greater dioxin concentration to elicit the same half-maximal effect.

pression (Hankinson, 2009; Sutter et al., 2009). The details of these effects are not sufficient to relate them to the much better studied effects in rat liver. Hence, to the extent of current knowledge, AHR activation is capable of altering cellular growth and homeostasis in both humans and animals. However, the specific effects on apoptosis inhibition observed in rat liver have not been observed in humans and the ductular reaction involving liver stem cell proliferation has not been observed in humans highly exposed to dioxin-like chemicals (e.g. Yucheng, Yusho, Viktor Yuschenko and the Vienna patients).

KE #3 – pre-neoplastic focal tissue changes

As noted, considerable hepatotoxicity in response to dioxin-like chemicals is observable in rats as histopathological changes that ultimately lead to liver tumors. In humans, there is no direct evidence linking sustained AHR activation to hepatotoxicity or liver cancer. In the Yucheng rice-oil poisoning incident, the rate of mortality from liver cancers was elevated whereas that from chronic liver disease was not (Tsai et al., 2007). In the Yusho rice-oil poisoning event, an increase in mortality from cirrhosis and chronic liver disease was observed, whereas cancers of the liver were not elevated (Onozuka et al., 2009). It is not known whether the liver diseases were due to causes other than dioxin-like chemicals, e.g. hepatitis viruses, alcohol consumption.

Both of these rice-oil poisoning episodes involved exposure to complex PCBs, polychlorinated dibenzofurans, quarterphenyl, and terphenyl compound mixtures, and conclusions regarding the causal relationship between the outcomes observed and sustained AHR activation is potentially confounded by the complex mixture exposures involved in the episodes. Even in chloracne cases, limited or no evidence of liver injury or even transient changes in liver enzyme levels, has been reported (Calvert et al., 1992; Geusau et al., 2005; Ghezzi et al., 1982; Mocarelli et al., 1986, 1991; Pocchiari et al., 1979; Reggiani, 1980).

Apical event/adverse outcome: liver cancer

Epidemiological evidence for human liver cancer is largely negative or at best equivocal (e.g. Seveso combined incidence and mortality: 17 observed/13.7 expected; trichlorophenol workers: 12 observed deaths/13.8 expected) (Boffetta et al., 2011). The major cause of cholangiocarcinomas worldwide is infection with the liver fluke *Chlonorchis sinensis*. In fact, this organism has been classified as "carcinogenic to humans" by IARC (Shin et al., 2010). *Chlonorchis sinensis* infection is endemic in parts of Asia and much less prevalent in Europe and North America. Cholangiocarcinomas have also been associated with primary schlerosing cholangitis, inflammatory bowel disease, type II diabetes, viral hepatitis, alcoholic liver disease, cigarette smoking and obesity (Cardinale et al., 2010; Welzel et al., 2007). Thorotrast, a contrast medium for X-ray diagnostics used between 1930 and 1950 that contains radioactive thorium, is also associated with cholangiocarcinoma (Jepsen et al., 2007). In sum, the apical outcome of liver tumors has not been observed in highly exposed workers and communities, suggesting that if liver tumor promotion is relevant for humans, it requires larger or more sustained exposures than occurred in these populations.

When the three KEs were considered individually in terms of their human relevance, the panel was not able to rule out the first KE since humans possess an AHR that is qualitatively similar to other model species and can be activated in a sustained manner. The panel was not able to rule out the second KE since human cell biology also possess signaling pathways (e.g. Wnt/β-catenin) associated with alterations in cellular growth and homeostasis. Although the third KE and the apical effect/adverse outcome have not been observed in humans and details of cell biology suggest humans are indeed less sensitive than rodents to liver injury, definitive evidence ruling out liver toxicity and carcinogenesis in humans is lacking.

Large quantitative differences between human and animal species exist for AHR binding affinity (Connor & Aylward, 2006), differential recruitment of coactivator proteins, and differential patterns of gene regulation (Flaveny et al., 2009, 2010); these differences suggest that humans are much less susceptible than animals to the hepatotoxic and carcinogenic effects of TCDD. In contrast, rodents are highly susceptible to the hepatotoxic and carcinogenic effects of TCDD

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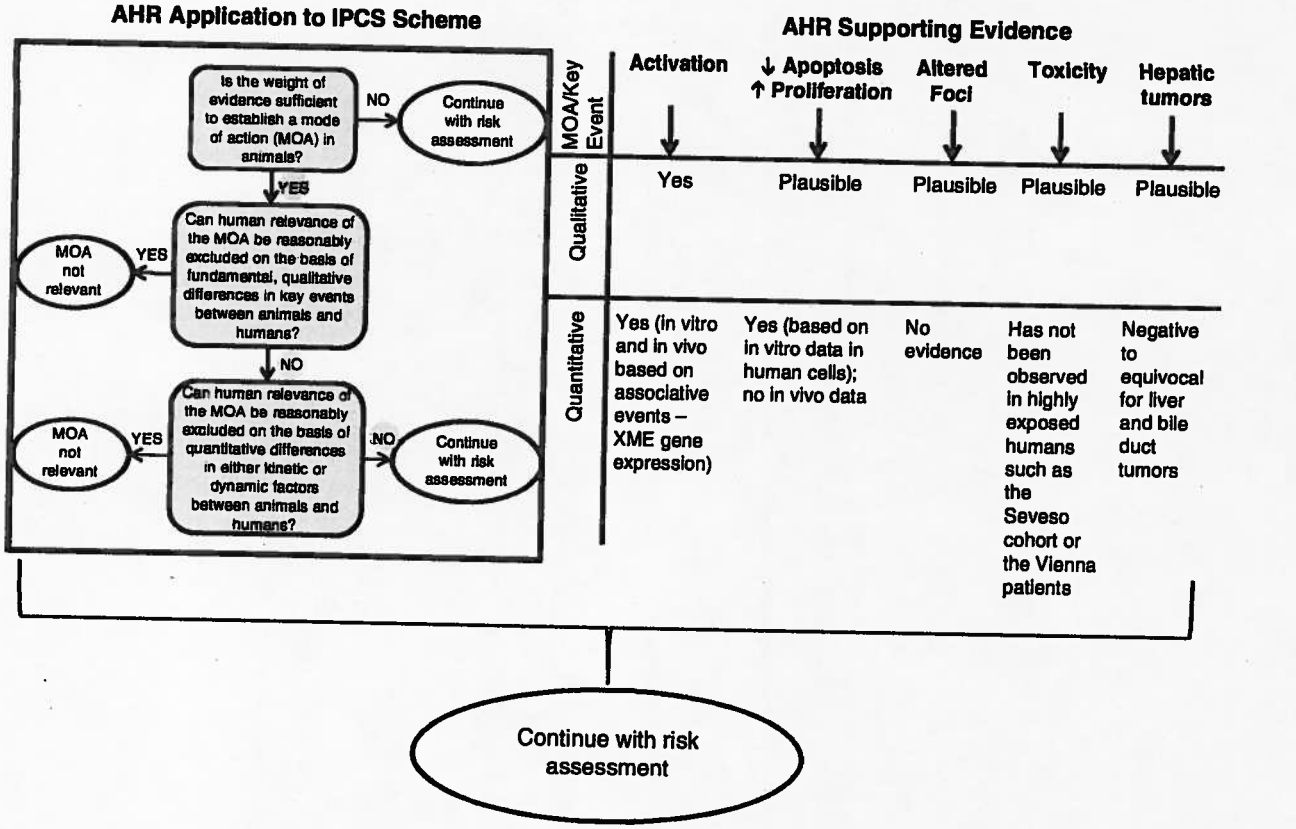

Figure 6. Application of the IPCS human relevance framework to the hypothesized AHR MOA. The adverse outcome and the key events closer to the apical outcomes (hepatocellular and cholangiolar cancers) have not been proven to occur in excess or occur at all, respectively, in humans exposed to relatively high levels of TCDD. However, the fundamental initial key event of AHR activation has been established to occur in humans following TCDD exposure resulting in CYP1A induction and chloracne.

(Hailey et al., 2005; NTP, 1980, 1982, 2006a,b,c,d). Thus, it is concluded that none of the KEs can be excluded as a relevant for humans, and based on the application of the IPCS framework, the human relevance of the proposed MOA cannot be reasonably excluded based on qualitative or quantitative evidence (Figure 6).

Dose–response and mode of action

Dose–response considerations for key events in the MOA

When the KEs in the MOA are placed in order of increasing severity from left to right, it becomes apparent that the estimated dose–response slopes (Hill function exponents or coefficients) for each event become steeper and the dose at which the event is first observed becomes greater. As the Hill coefficient increases, the slope becomes steeper and more non-linear, reflecting the dose–response relationships supporting the proposed sequence of KEs (i.e. the Hill consideration of dose–response concordance is supported). Figure 4 shows this for a number of events measured in NTP studies (NTP, 2006a,b,c,d). Xenobiotic metabolizing enzyme induction is the earliest effect and occurs at all doses. If Hill functions are fit to measures of xenobiotic metabolizing enzyme induction such as EROD, PROD or A4H, the Hill coefficients are all ~1 and the half maximal values that occur at a liver concentration between 1000 and 2000 ng/kg. The value of the Hill coefficients reflect the complexity of molecular interactions, with larger Hill coefficient values arising from more complex interactions.

One way to think of downstream KEs is as a consequence of early AHR activation combining with other later events, resulting in the more apical events exhibiting very steep dose–response curves and large-valued Hill coefficients. However, CYP induction within hepatic zones or within single hepatocytes may also show high Hill coefficients. There may be mechanisms that alter the Hill coefficient within cells or lobule regions and in some instances may even result in oscillatory behavior of the system (Matthews et al., 2005; Nguyen & Kulasiri, 2009). Therefore, another explanation is that increasing Hill coefficients observed for more distal KEs in the MOA may reflect induction in the least sensitive portion of the liver, i.e. the periportal zone. An example of such a steep late event would be toxic hepatopathy and the associated cascade of inflammatory cytokines or bile duct hyperplasia (Figure 7).

Relatively early events in the MOA associated with hepatocellular adenoma are hepatocyte hypertrophy, multinucleated hepatocytes and diffuse fatty change. These have Hill coefficients between 1.5 and 3 and half maximal values at 104-week average liver TCDD concentrations between 5000 and 10 000 ng/kg (Figure 8a). The two events related to cholangiocarcinoma are bile duct hyperplasia and oval cell hyperplasia, which are relatively late occurring events. They have steeper slopes (i.e. greater Hill coefficients) than the precursor events related to hepatocellular adenoma and half

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maximal values around a 104-week average liver concentration of 7000 ng/kg (Figure 8b and Figure 4). Cell division as measured by BrdU labeling index at 31 weeks has a Hill coefficient of almost three and a half-maximum value at a liver concentration of almost 15 000 ng/kg. An important question is how the Hill coefficients for these KEs compare to the Hill coefficient estimates for hepatocellular and cholangiolar cancer development. The two apical events have still steeper slopes; the Hill coefficients are over 3 for both tumor types. The half maximal concentration as a 104-week average liver concentration for cholangiocarcinoma is ~15 000 ng/kg and that for hepatocellular adenoma is ~20 000 ng/kg.

The frequencies of various KEs in the MOA increase with both dose and time. This can be seen in Figure 3 which shows the increase in the volume fraction of GSTP altered hepatic foci with both dose and time over a 6-month period. The same type of effect can be seen in Figure 7 for toxic hepatopathy and bile duct hyperplasia from the NTP bioassay (NTP, 2006a,b,c,d), and increases along both the dose and time axes occur for both these events. EPA Guidelines for Carcinogen Risk Assessment (USEPA, 2005) indicate that precursor events can be used to extend the dose–response curve to lower doses. The non-apical events have lower slopes and lower half-maximal values (Figure 4). Thus, the point of departure for non-apical dose–response curves is to the left (lower dose) of the apical point of departure, and therefore a BMD or BMDL used as the basis for a toxicity criterion is more protective of the apical endpoint. The use of non-apical endpoints to develop a toxicity criterion protective of the apical endpoint is similar to the approach taken by EPA in the risk assessment for chloroform (USEPA, 2001). The increasingly steeper slopes associated with more apical events also have important risk assessment implications since the margin of safety provided at a particular BMD or BMDL is much wider when the dose–response is steep than when based on a lower slope.

A recent statistical analysis of cancer potency estimates developed from the dose-dependent incidence of hyperplastic nodules and hepatocellular carcinoma (Kociba et al., 1978) calculated BMD_{10} values of 20.72 and 7.89 ng/kg day (Shao & Small, 2011). The same study calculated BMD_{10} values of 46.82 and 24.22 ng/kg day for the dose-dependent incidence of neoplastic nodules (from NTP, 1982). While these values were developed from different studies than NTP (2006a,b,c,d), the median of these four values is 22.5 ng/kg day, very close to the dose at which tumors first appear in the NTP TCDD bioassay (NTP, 2006a).

Initiation–promotion assays provide extensive data for quantitative dose–response modeling, assuming histologically observed cell proliferation (e.g. volume fraction increase) reflects increased cell survival occurring from intrafocal inhibition of apoptosis (cell proliferation in the absence of increased cell division) (Chopra & Schrenk, 2011; Luebeck et al., 2000; Schrenk et al., 1994; Stinchcombe et al., 1995; Teeguarden et al., 1999) (Figure 3). Viluksela et al. (2000) observed an apparent threshold in the dose-dependent increase in volume fraction of GSTP+ foci in both the TCDD-sensitive Long-Evans rats and TCDD-resistant Han–Wistar rats. The Han–Wistar strain required a higher TCDD dosage to achieve a comparable increase in volume fraction and the number of GSTP+ foci. Increased cell division has been demonstrated in the NTP cancer bioassays (NTP, 2006a,b,c,d) and in a number of the initiation–promotion studies. However, these increases in cell division appear to require longer periods of sustained AHR activation than elicited changes in apoptosis.

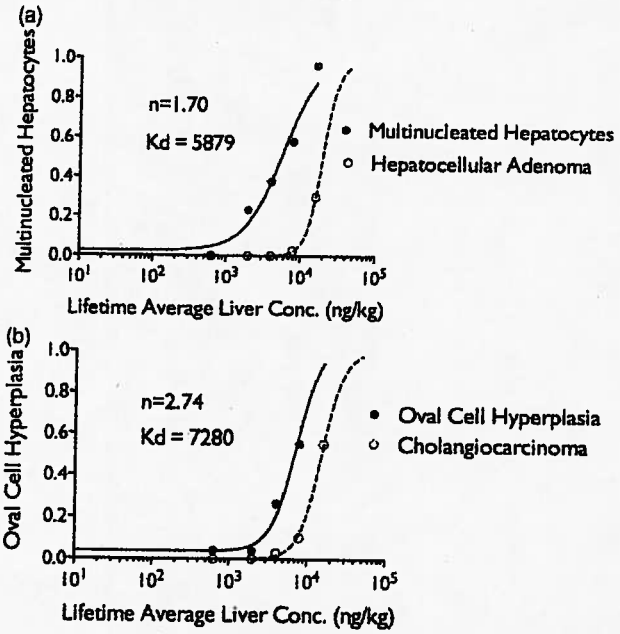

Figure 7. (a and b) Comparison of the dose–response of key and apical events. Upper panel (a): Dose–response for multinucleated hepatocytes and hepatocellular adenomas as a function of lifetime average liver concentrations of TCDD. Lower panel (b): Dose–response for oval cell hyperplasia and cholangiocarcinoma. In both cases, the identified KE could serve as a precursor for risk assessment purposes. In both plots, the Hill coefficient (n) and the half-maximal value (Kd) are shown for the precursor KE.

Quantitative dose–response modeling of AHR activation

It is feasible to develop quantitative dose–response models of the AHR pathway that incorporate the large number of genes altered by TCDD (Boverhof et al., 2006; Carlson et al., 2009). The bulk of the transcriptional data are based on CYP1A1 induction, arguably the most sensitive biomarker of AHR activation. If CYP1A1 induction is chosen as the basis of a dioxin risk assessment, care needs to be exercised not to exaggerate the potential human cancer risks from dioxin. This is important because CYP1A1 induction is a marker for "activation" of the AHR, but not necessarily a marker for "sustained activation", as noted in the proposed MOA (e.g. exposures can product transient increases in CYP1A1). In this regard, Simon et al. (2009) showed that a BMD based on EROD enzyme induction from NTP (2006a,b,c,d) was 6- to 10-fold lower than a BMD based on tumor occurrence. Should transcriptional responses be used to anchor the lower bound of the dose range for cancer risk, a number of challenges will need to be met. These include the use of human data and genomics, *in vitro* to *in vivo* extrapolation, novel uses of the margin of exposure concept, and likely others (Judson et al., 2011; Rotroff et al., 2010; Thomas et al., 2011).

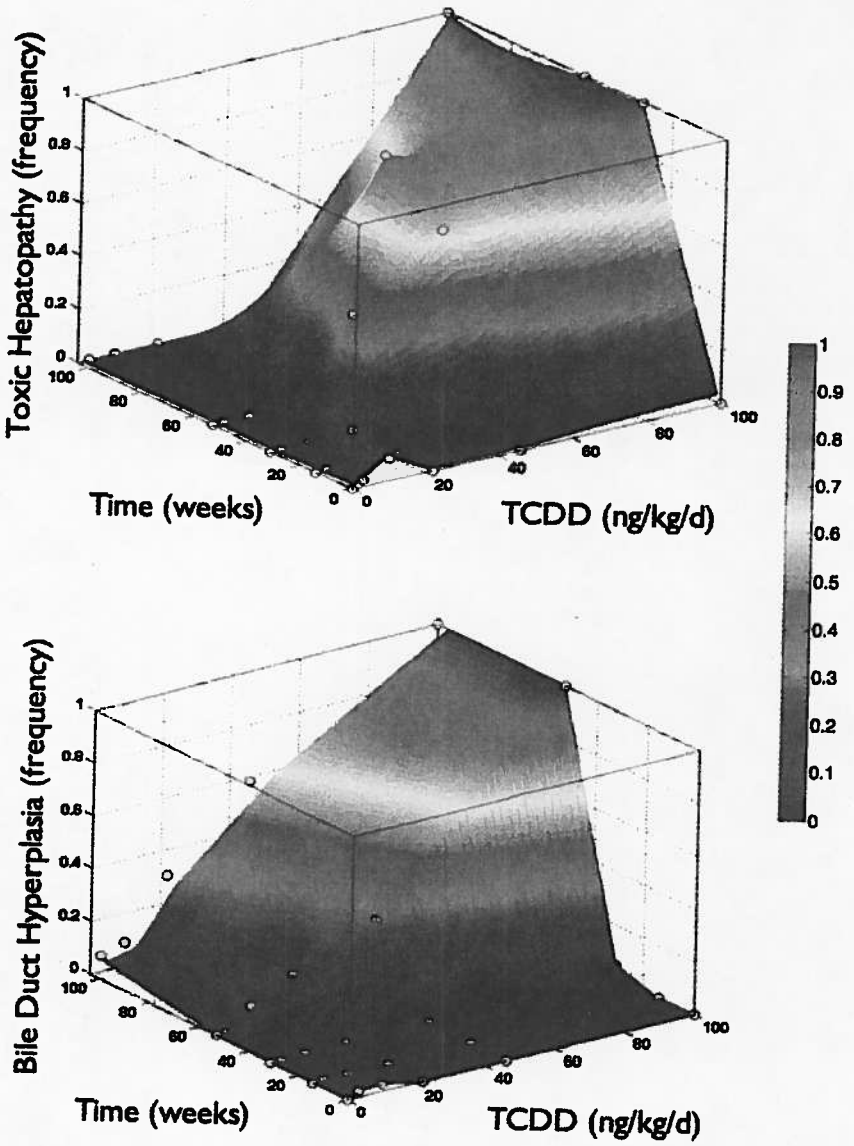

Figure 8. Dose- and time-dependence of the occurrence of toxic hepatopathy and bile duct hyperplasia in rats from NTP (2006a,b,c,d). The data were severity-adjusted frequencies (Simon et al., 2009) at 14, 31, 53 and 104 weeks and are shown as gray circles embedded in the surface responses of toxic hepatopathy and bile duct hyperplasia. The corresponding interpolated surfaces are shown in false colors with the color coding shown to the right. Values >0.7 for both events only occur late and at relatively high doses.

When sufficiently low doses or concentrations are used, CYP1A induction may appear to be non-linear and even have an observable induction threshold. Van den Heuvel et al. (1994) observed two-site (i.e. non-linear) kinetics of binding of activated AHR to AHREs. There was no increase in CYP1A1 mRNA observed in the low dose region on a whole-liver basis, though this was interpreted as possibly being due to a pool of non-degradable mRNA obscuring observation of *de novo* mRNA at low concentrations. Over the entire dose range, induction appeared nearly hyperbolic, thus following a first order Hill function (i.e. the Hill coefficient, *n* equal to 1). Andersen et al. (1997) demonstrated that with four to five zones along the sinusoid it was possible to have highly non-linear induction in each zone but apparent consistency with a first-order Hill relationship over the whole tissue due to regional differences in effective "Kd" or half-maximal binding affinities between the zones. However, Budinsky et al. (2010) observed Hill coefficients close to unity for CYP1A transcriptional responses to dioxin in primary hepatocytes in culture. The transcriptional responses observed were averages across a large number of cells that were presumably derived from various regions of the liver and this may limit an evaluation of the apparent zonal-dependency of AHR activation as a ModF using *in vitro* methods.

Consideration of alternative modes of action

Alternate MOA(s) must be examined as part of the MOA and human relevance framework approach (Boobis et al., 2006, 2009; Cohen et al., 2003, 2004; Holsapple et al., 2006; Julien et al., 2009; Meek, 2008; Meek et al., 2003; Seed et al., 2005; Sonich-Mullin et al., 2001; USEPA, 2005). The expert panel concluded that the MOA for liver carcinogenesis is not due to direct acting genotoxicity, but rather via sustained AHR activation and is most likely to be non-linear. The evidence for this has been discussed earlier. The expert panel reviewed the relevance of oxidative stress and ROS production as alternative MOAs (see discussion earlier under ModFs) since they can be linked to mutagenicity and genotoxicity. Potential mutagenicity of TCDD, although unlikely, was considered as an alternative MOA because of the potential to alter the low-dose extrapolation method and thus, risk assessment results. As discussed, no evidence for DNA adducts formed by catechol estrogens binding to DNA was found using a sensitive

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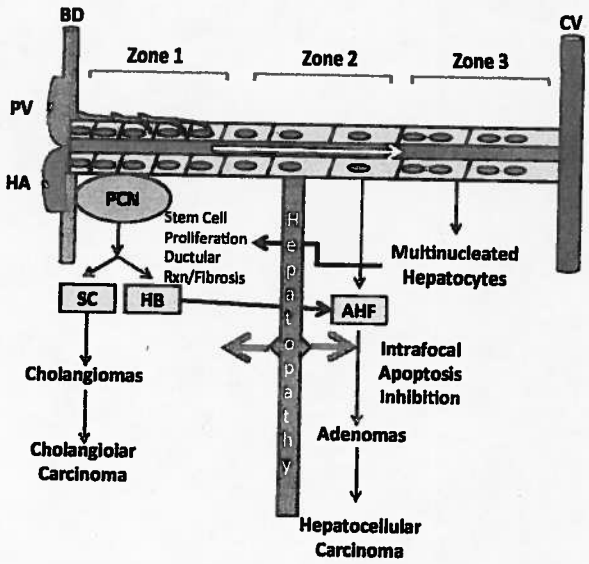

Figure 9. In this figure, the basic structure of the liver acinus is depicted (BD: bile duct; HA: hepatic artery; PV: portal vein; CV: central vein; SC: small cholangiocytes; HB: hepatoblasts; AHF: altered hepatic foci; PCN: pluripotential cell niche). From left to right, the figure shows the portal triad and the hepatocytes that change from the more nascent, oval-cell like hepatocytes (smaller cells with a smaller cytoplasm/nucleus ratio) to the older, larger, polyploidal cells near the central vein. The zones of the acinus are also shown. Inserted into this graphic are the aspects of liver cancer biology including the role of the stem cell as a source of both bile duct and liver cell tumors and the source of liver tumors from more mature hepatocytes that can contribute to altered hepatic foci. Interspersed in this figure are elements of the proposed dioxin/AHR MOA, KE(s), AE(s) and ModF(s) – see text for more discussion.

accelerator mass spectrometry method (Turteltaub et al., 1990). Endogenous DNA adducts (I compounds) were reduced following sub-chronic TCDD treatment (Randerath et al., 1990).

The panel agreed that the weight of evidence, including a number of mutagenicity and genotoxicity studies of dioxin-like chemicals, suggest that these chemicals are not mutagenic (Bock & Kohle, 2005, 2006; Cohen, 1998; Dragan & Schrenk, 2000; Knerr & Schrenk, 2006; Schwarz et al., 2000; Whysner & Williams, 1996). This conclusion is consistent with recent regulatory assessments of dioxins. A framework for the consideration of DNA adducts as part of a carcinogenic MOA has been developed (Himmelstein et al., 2009; Jarabek et al., 2009; Swenberg et al., 2008, 2011). Examination of potential KEs in the MOA that could be related to DNA damage induced by dioxin, the formation of DNA adducts or mutations within the context of this framework, could not confirm a DNA-reactive or mutagenic MOA for dioxin.

Discussion

Overall conclusions about the MOA of dioxin involving the AHR

The workshop and the AHR panel were able to establish a MOA with defined KEs, AEs and ModFs (Figure 2). This MOA can be used not only in risk assessment efforts for dioxin-like chemicals, but also to examine dose–response modeling. With knowledge of the events in the MOA and the range of doses or tissue concentrations associated with each event, we will be better able to determine the overall dose–response range corresponding to a defined risk level for liver tumor promotion.

Building from the analyses developed at the meeting, a variety of quantitative dose–response assessments for KEs and AEs within the tumor promotion MOA of TCDD can be described. As the MOA progresses toward tumors, these events represent increasingly complex biological changes. This complexity is reflected in the histopathological findings (Goodman & Sauer, 1992; Hailey et al., 2005). As the MOA progresses toward tumors, Hill model fits of the KEs and AEs tend to show increasing Hill coefficients and increasing half-maximal concentrations, indicating an increasing trend toward non-linearity and the requirement for higher doses. The largest Hill coefficient and half-maximal concentrations occur for the apical events of hepatocellular and biliary cancer.

Relationship of proposed MOA and biological models for liver tumorigenesis

Figure 9 depicts a biological model for liver cancer along with some of the MOA, KE, AE and ModF elements for dioxin and the AHR (Alison, 2005; Furuyama et al., 2011; Gaudio et al., 2009; Greenbaum & Wells, 2011; Lefkowitch, 2009; Sell & Dunsford, 1989; Sempoux et al., 2011; Turner et al., 2011). As shown in Figure 9, the concept of the "streaming liver" includes not only the movement of material from the portal vein to the central vein and of bile in the opposite direction, but also the movements of cells formed in progenitor cell niches. Newly formed hepatoblasts become new hepatocytes and move from the periportal region toward the centrilobular region, from Zone 1 toward Zone 3. On the way, they increase in ploidy and some may become multinucleate (Duncan et al., 2010; Scott et al., 1989; Seglen, 1997). Liver stem cells/oval cells located in the progenitor cell niches also give rise to

precursor cholangiocytes that move periportally to become the epithelial cells of the small bile ducts.

Understanding this liver cancer biology helps to elucidate MOA and data gaps. Hepatocytes residing in the centrilobular region of the lobule (Zone 3) are the most sensitive to AHR activation by TCDD in terms of CYP1A induction. These centrilobular hepatocytes are presumed to be the "oldest" hepatocytes (Andersen et al., 1997; Tritscher et al., 1992). Whether differences in AHR activation exist between polyploid hepatocytes and mature diploid hepatocytes is not known; however, these sensitive hepatocytes, respond to TCDD with a G1-S cell cycle delay (Bock & Kohle, 2005; Mitchell et al., 2006). This delay would provide a growth stimulus to hepatocytes capable of cell division and to liver stem cells. Sustained AHR activation leading to an excessive proliferatory signal would increase division of both hepatocytes and liver stem cells in the progenitor cell niches. Growth of stem cells may be accompanied by the ductular reaction and the development of biliary fibrosis (Fausto, 2004; Glaser et al., 2009; Oh & Petersen, 2003; Petersen, 2001). Dose–response modeling suggests that this growth stimulus affects hepatocytes before it affects stem cells – at earlier times and at lower doses (Figure 9).

The progenitor cell of each focus may originate from a dividing hepatocyte, hepatoblast or an oval cell moving from the progenitor cell niches toward Zone 3 (Fausto, 2004; Goldfarb et al., 1983; Kuhlmann & Peschke, 2006; Tsuji et al., 1988). In the mid zone region (Zone 2), altered hepatic foci would occur when single cells acquire sufficient mutations to become initiated and grow into a focus. In rats, the spontaneous formation of altered hepatic foci from these initiated mid zonal cells is a slow process (Harada et al., 1989; McMartin et al., 1992; Newsholme & Fish, 1994; Popp et al., 1985; Ward & Henneman, 1990). Altered foci may also form from centrilobular diploid hepatocytes and older polyploid hepatocytes (Sarafoff et al., 1986).

Sustained AHR activation leads to inhibition of intrafocal apoptosis that allows the foci to escape destruction, grow in volume and potentially acquire additional mutations. In addition to inhibition of apoptosis, a mitogenic stimulus is created by subsequent hepatopathy that could also facilitate the growth of altered hepatic foci. This hypothesis is supported by the observation of increasing BrdU labeling with time over 12 months of TCDD administration in the NTP studies (Hailey et al., 2005).

In the periportal region (Zone 1), emerging hepatoblasts and newly formed hepatocytes and cholangiocytes may become initiated. These presumptively tumorigenic cells would receive the same proliferative stimuli as altered cells within foci in Zones 2 and 3, which includes the growth stimulus caused by G1-S cell cycle delay occurring in mature hepatocytes and the mitogenic stimulus resulting from hepatopathy. However, unlike the slower growing foci in the parenchyma, initiated cells in Zone 1 likely have longer lifespans. In fact, with higher doses of TCDD, cell division is observed in Zone 1 with BrdU labeling and from histopathological examination showing oval cell and bile duct hyperplasia (Hailey et al., 2005; Maronpot et al., 1993) that, in the presence of sustained proliferative stimuli, produces cholangiofibrosis and cholangiocarcinoma.

Overall the functional anatomy and interactions of cell subtype structures in the liver are very consistent with the proposed MOA. The proposed KEs, AEs and ModFs identified by the AHR panel are consistent overall with the known biological and clinical features of liver cancer development, and fulfill the consideration of biological plausibility (Boobis et al., 2009; Hill, 1965). USEPA (2005) makes a clear distinction between "mode of action" and "mechanism of action" in terms of the level of detail of the available knowledge. That distinction is useful here and, to summarize, the extant knowledge of cancer biology in the liver is more than sufficient to support the mode of action and the identification of KEs, AEs and ModFs, even though detailed mechanistic knowledge of the entire sequence of events from sustained AHR activation to tumor formation is not yet available.

Considerations of the proposed MOA for dose–response analyses

Use of early effect biomarkers for dose–response

The analyses of AHR-mediated tumor response as developed by the expert panel provides an excellent platform for exploring the utility of alternative methods for better incorporating early effects into decision-making regarding likely dose–response behavior to support environmental risk assessments. A common practice in assessing the biological risk of low-dose chemical exposures is to extrapolate linearly from higher doses where effects are more reliably measured. As described in this article, a systematic evaluation of KEs for the proposed MOA for the AHR can be used to directly evaluate assumptions about low dose–response behavior. This application of the early effects data is consistent with recent recommendations regarding enhancements to the current IPCS MOA framework (Boobis et al., 2009) and the vision for greater use of systems biology approaches in the assessment of chemical toxicity (NAS, 2007).

Linear versus non-linear

The complex cascade of protein interactions that arise from AHR-ligand interactions at the detailed mechanistic level are often represented as a relatively simple bi-molecular interaction. Ligand binding and the constellation of early steps in gene transcription have Hill coefficients close to unity and thus, their dose–response may be presumed to be linear (McGrath et al., 1995; Murrell et al., 1998; USEPA, 2003). The outcome of the initial KE, sustained AHR activation, results in the constellation of more complex effects termed hepatopathy, as well as effects on cell division and apoptosis. These subsequent events represent complex phenomena that depend on both dose and time, and are highly non-linear. There is sufficient information regarding the MOA that some of the KEs or AEs could be selected as precursor events and used to develop regulatory toxicity criteria (USEPA, 2005).

With regard to the use of KEs or AEs as precursors, Dellarco and Baetcke indicate:

> Mode of action data have come into play in several ways in EPA risk assessments. It has been critical in informing the

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dose–response relationship below the experimental observable range of tumors, and thus it is useful in establishing more appropriate guidance levels for environmental contaminants (Dellarco & Baetcke, 2005).

In this regard, Simon et al. (2009) explored the use of precursor events for a risk assessment of TCDD based on the NTP studies (NTP, 2006a,b,c,d). They also showed that tumors did not occur in the absence of hepatopathy. Figure 5(a) and (b) indicate that an increase to ~70% of the maximum in EROD induction occurs at a dose of 22 ng/kg day where tumors first appear.

Additivity to background and population variability

Two issues related to the linear non-threshold versus non-linear threshold are: (1) the hypothesis of additivity to background and (2) the possibility that populations exhibit widely ranging individual thresholds thereby yielding population distributions whose lower ends are essentially zero (Hattis, 1996; Lutz, 2001; Lutz & Gaylor, 2008; Lutz et al., 2006). Some have interpreted these issues to mean that biological knowledge is incapable of providing useful information for risk assessment at very low doses (Crump et al., 2010). These issues were purposely avoided during the workshop so that the panel could focus on the MOA itself and the lessons one can garner for dose–response behavior based solely on the biology of the AHR. Future discussions should explore the role of biological knowledge in risk assessment and whether or not background additivity and/or variation in population susceptibility are supported by the available science and also whether these issues are germane to regulatory policy (Rhomberg et al., 2011; Simon, 2010).

Next steps in elucidating the MOA

The proposed MOA is clearly sufficient for establishing a basis for dose–response modeling of KEs and AEs while taking into account important ModFs. However, many questions remain over how the many aspects of the KEs, AEs and ModFs interactively combine to produce tumor promotion resulting in hepatocellular adenomas and carcinomas, cholangiomas and cholangiolar carcinomas. These questions largely relate to the three or possibly four types of liver cells that give rise to hepatocarcinogenesis (hepatocytes, stem/oval cells, bile duct cells, periductular cells, reviewed in Alison, 2005). For example, how does the G1-S cell cycle block induced by AHR activation in normal hepatocytes induce both hepatocytes and liver stem cells into cell division and differentiation with the increased likelihood for mutations and possible neoplastic transformation? Do foci derived from mature polyploid and possibly multinucleate, hepatocytes contribute in a different way to tumor formation than foci derived from younger diploid hepatocytes? Do the relatively benign adenomas arise from both these types of foci or only from a single type?

The MOA likely involves changes in stem cells as well as early pathway disruptions. Future research into non-parenchymal cells, especially the liver stem cells, will further illuminate both the qualitative and quantitative aspects of the MOA proposed in this article. Likewise, better quantitative assessment is needed for the KE of inhibition of intrafocal apoptosis. A better understanding of low-dose responses where genes are switched on over a narrow range of ligand concentrations is needed, as well as clarification of the suite of gene transcription changes that drive the apical effects. Such knowledge would allow a transition from use of dose–response data for associative events (such as CYP induction) to changes that are directly linked to downstream key events, which may have different dose–response characteristics. This non-linear transcriptional response is characteristic of auto-regulatory feedback loops and is a mechanism common among other nuclear receptor proteins (Andersen & Barton, 1999; Zhang & Andersen, 2007). High-throughput screening and bioinformatic tools, such as those being developed as part of the Tox21 collaboration, will help to uncover functions and connections among gene networks to produce more mechanistic dose–response models. The development of computational methodologies for identifying gene signatures, particularly those that dampen transcriptional activation through negative feedback loops, should be useful in the identification of genes controlled by nuclear receptors. How and when will the relevant information be developed and how will it impact the dose–response modeling available with the current data? As the information evolves, the MOA will be refined. With these expected refinements and concomitant growth of knowledge of the role of the AHR in health and disease, one also expects increased opportunities for targeting the AHR for disease prevention and therapy.

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Declaration of interest

This manuscript, including all analyses, interpretations and opinions expressed are exclusively those of the authors and are not necessarily those of their employers or respective institutions, as indicated on the cover page. This article may be the work product of an employee or group of employees of the NIEHS, National Institutes of Health (NIH); however, the statements, opinions or conclusions contained therein do not necessarily represent the statements, opinions or conclusions of NIEHS. This article reflects the views of the authors and

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Supplementary material available online

Supplementary Summary Tables
Supplementary Figure